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Immunoregulation mediated by cell surface sulphydryl groups in rheumatoid arthritis

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IMMUNOREGULATION MEDIATED BY CELL SURFACE SULPHYDRYL GROUPS
IN RHEUMATOID ARTHRITIS

Submitted by

Catherine Helen Brown-Galatola B.Sc. (Hons.) AIMLS

for the Degree of Doctor of Philosophy
of the University of Bath

1989

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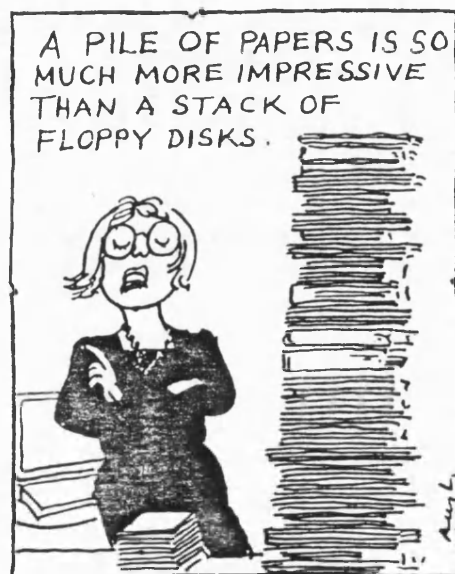
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DEDICATIONS

This thesis is dedicated to my family and my husband, Dr. Giovanni Galatola, for their love, encouragement and practical help and support throughout the gruelling experience! It is also dedicated to my late grandfather, Mr. George Craine (1902-1986).

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CONTENTS

	PAGE No
TITLE.....	i
CARTOON.....	ii
DEDICATIONS.....	iii
ACKNOWLEDGEMENTS.....	iv
CONTENTS.....	v
SUMMARY.....	ix
ABBREVIATIONS.....	xi
CHAPTER 1.1: INTRODUCTION.....	1
Historical background.....	1
Rheumatoid arthritis.....	2
The musculoskeletal manifestations of RA.....	5
Extraarticular manifestations of RA.....	6
The normal synovial joint structure.....	9
Pathology of the rheumatoid joint.....	13
Pathogenesis of RA.....	20
Immunoregulation.....	41
In vitro functional models for suppressor cell activity.....	78
Aetiology of RA.....	92
Immunogenetics.....	95
Chemotherapy in RA.....	98
Patient management.....	107
The role of the SH group in RA.....	108

Ankylosing spondylitis.....	133
Psoriatic arthritis.....	134
Systemic lupus erythematosus.....	135
CHAPTER 1.2: AIMS.....	139
CHAPTER 2.1 MATERIALS.....	141
CHAPTER 2.2 EQUIPMENT.....	145
CHAPTER 3: METHODS.....	149
CHAPTER 4: RESULTS.....	198
4.1 Comparison of serum thiol levels in various patient groups and normals.....	198
4.2 Phenotypic comparison of PBMNC in RA NSAID and normals.....	200
4.3 Setting up the ELISA assay.....	201
4.4 Effect of PHMPSA on IgG synthesis.....	205
4.5 Comparison of IgG and IgM synthesis in various patient groups and normals.....	207
4.6 Effect of 2-ME on IgG and IgM synthesis in various patient groups and normals.....	209
4.7 Effect of H ₂ O ₂ on IgG synthesis.....	210
4.8 Setting up the CON-A induced suppressor cell assay for suppression of Ig synthesis.....	211
4.9 Comparison of CON-A induced suppressor cell activity in various patient groups and normals.....	216

4.10 Effect of D-PEN treatment "in vitro" on CON-A induced suppressor cell activity in RA NSAID..	223
4.11 Setting up the short-lived suppressor cell assay.....	225
4.12 Comparison of SI in various patient groups and normals.....	225
4.13 Effect of PHMPA on "induction" and "activity" of CON-A induced suppressor cell expression in normals.....	227
4.14 Effect of 2-ME treatment on CON-A induced suppression in RA NSAID patients.....	231
4.15 Effect of H ₂ O ₂ on CON-A induced suppression...	234
4.16 Effect of PHMPA on mitogen stimulated proliferation.....	237
4.17 Effect of 2-ME treatment on proliferation of RA NSAID cells.....	238
4.18 Effect of H ₂ O ₂ on mitogen stimulated proliferation.....	239
4.19 Production of "educated " cells for IL-2 estimation.....	241
4.20 Effect of PHMPA on IL-2 production.....	243
4.21 Comparison of IL-2 production in various patient groups and normals.....	244
4.22 Effect of PHMPA on cell surface antigen expression.....	247

4.23 Effect of PHMPA on ability of "educated"	
cells to proliferate with r-IL-2.....	251
4.24 Effect of PHMPA on ability of r-IL-2 to	
bind to the IL-2 receptor.....	256
4.25 Effect of PHMPA on superoxide production	
from neutrophils.....	261
CHAPTER 5.1: DISCUSSION.....	263
CHAPTER 5.2: CONCLUSION.....	344
CHAPTER 5.3: FUTURE AIMS.....	348
REFERENCES FOR CHAPTER 1.....	354
REFERENCES FOR CHAPTER 3.....	411
REFERENCES FOR CHAPTER 4.....	419
REFERENCES FOR CHAPTER 5.....	425
PUBLICATIONS.....	455

N.B. Figures and tables were allocated two numbers (i.e. 4.121), the first refers to the chapter, the second according to the order in which they are quoted within the chapter. References are numbered consecutively within each chapter.

SUMMARY

Rheumatoid arthritis (RA) is an autoimmune disease characterised by a derangement of immunoregulatory mechanisms in the immune system and also by reduced thiol (-SH) levels on cell surface proteins and soluble proteins. The latter probably results from the inflammatory nature of the disease which causes production of reactive oxygen species (ROS) from phagocytic cells. We questioned what aspects of the immunoregulatory cell circuits could be -SH dependent, and whether oxidation of important cell surface -SH groups could account for the immunological abnormalities observed in RA and the consequent clinical improvement after treatment with sulphhydrate drugs (D-Penicillamine, Aurothiomalate, etc.).

In healthy controls Concanavalin-A induced suppressor "activity" but not its "induction", was found to be inhibited following para-hydroxymercuriphenylsulphonic acid (PHMPSA - an irreversible surface -SH blocker) incubation, and RA patients on NSAID showed impaired CON-A induced suppressor activity for suppression of B-cell responses, which could be improved in vitro by 2-Mercaptoethanol (2-ME - a -SH reducing agent) or sulphhydrate drugs in vivo. Defective CON-A induced suppression of T-cell responses, improvable by 2-ME treatment was also noted in four RA NSAID patients.

T-cell proliferation in response to CON-A, PHA, PWM, or anti-CD3 Mab, was found to be inhibited by prior treatment with PHMPSA, as was proliferation of "educated" cells in response to rIL-2, so too was proliferation on treatment with PHMPSA after production of "educated"

cells.

The expression of various T-cell antigens involved in immunoregulation (e.g. CD2, CD3, CD4, CD8, CD45R, CDW29 and other surface Ag - e.g. CD11, CD57, CD37), and both the induction and the expression of CD25 and HLA-DR were not inhibited by PHMPSA treatment. PHMPSA did not inhibit IL-2 production from normals and 2-ME did not increase IL-2 production in RA NSAID, which was not significantly different from normals. 2-ME did however improve proliferation of RA cells in response to mitogens. The ability of ^{125}I -IL-2 to bind to the IL-2 receptor was slightly inhibited by PHMPSA pretreatment, likewise receptor mediated (FMLP) but not non-receptor mediated (PMA) stimulation of superoxide production in neutrophils was thiol dependent, perhaps, as for the IL-2 receptor by PHMPSA inhibiting FMLP binding or alternatively inhibiting signal transduction.

Results from experiments carried out with H_2O_2 (a physiological oxidising agent) paralleled those using PHMPSA.

A relationship was hypothesised between raised levels of ROS leading to reduced cell surface -SH levels and subsequently defective suppressor cell activity. Hypofunction in various suppressor cell circuits (short lived suppressor cell activity, CON-A induced suppression of T- and B-cell responses) vitally important in immunoregulation was noted in RA NSAID, and these could play a significant role in both the immunopathogenesis and/or perpetuation of RA.

ABBREVIATIONS

AA	Arachidonic acid
Ab	Antibody, Antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen(s)
ALMR	Autologous mixed lymphocyte reaction
ANA	Antinuclear antibody
AS	Ankylosing spondilitis
Au	Aurothiomalate
BCDF	B-cell differentiation factor
BCGF	B-cell growth factor
BSA	Bovine serum albumin
C'	Complement
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CON-A	Concanavalin A
CP	Caeruloplasmin
CRI	Cross-reactive idiotype
CRP	C-reactive protein
D.D.	Disease duration
DIP	Distal interphalangeal joint(s)
D-PEN	D-Penicillamine
EA(D)	Extra-articular (disease)
EBV	Ebstein-Barr virus
ELISA	Enzyme-linked immunosorbent asaay
EMS	Early morning stiffness

ISR	Erythrocyte sedimentation rate
IACS	Fluorescence activated cell sorter
IcR	Fc receptor
ILMP	n-Formyl-1-methionyl-1-leucyl-1-phenylalanine
glutathione	Unless otherwise stated means "reduced glutathione"
GSH	Reduced glutathione
GSSG	Oxidised glutathione
γ -IFN	γ -interferon
HAGG	Heat aggregated IgG
H/I	Helper/inducer cell(s)
IBF	Immunoglobulin binding factor
ICs	Immune complexes
id	Idiotypic
IEP	Immuno-electrophoresis
Ig	Immunoglobulin(s)
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IP	Interphalangeal joint(s)
LGL	Large granular lymphocyte(s)
LPS	Lipopolysaccharide
L ₇	Leukotriene
LØ	Lymphocyte(s)
Mab	Monoclonal antibody, Monoclonal antibodies
MCP	Metacarpophalangeal joint(s)
MNC	Mononuclear cell(s)
MLR	Mixed lymphocyte reaction

MTP	Metatarsophalangeal joint(s)
MØ	Monocyte(s)/macrophage(s)
NK	Natural killer cell(s)
NSAID	Non-steroidal anti-inflammatory drug(s)
OA	Osteoarthritis
PB	Peripheral blood
PBMNC	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PFC	Plaque forming cell(s)
PG	Prostaglandin(s)
PHMPSA	Para-hydroxymercuriphenylsulfonic acid
PIP	Proximal interphalangeal joint(s)
PLTS	Platelets
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leucocyte(s)
PSA	Psoriatic arthritis
PVC	Polyvinyl chloride
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RANA	Rheumatoid arthritis nuclear antigen
RF -ve (+ve)	Rheumatoid factor negative (positive)
ROS	Reactive oxygen species
SA	Staphylococcus aureus
SBF	Suppressor B-cell factor
SF	Synovial fluid
SFL	Synovial fluid lymphocyte(s)
SLE	Systemic lupus erithematosus

S/I	Suppressor/inducer cell(s)
sIG	Surface immunoglobulin(s)
SLSA	Short-lived suppressor assay
SM	Synovial membrane
SRBC	Sheep red blood cells
SRS-A	Slow reacting substance of anaphylaxis
ST	Synovial tissue
T _C	T-cytotoxic cell(s)
T _H	T-helper cell(s)
TNS	Tumour necrosis serum
TRF	T-cell replacing factor
T _S	T-suppressor cell(s)
T _{S/C}	T-suppressor/cytotoxic cell(s)
WBC	White blood cell(s)
2-ME	2-mercaptoethanol
5HETE	5-hydroxyeicosatetreinoic acid
5HPETE	5-hydroxyperoxyeicosatetreinoic acid
UNT	Untreated

CHAPTER 1.1 - INTRODUCTION

HISTORICAL BACKGROUND

Rheumatic disorders have been recognized since the fifth century B.C. usually passing under the general term "arthritis" (Greek arthron = joint, itis = inflammation) meaning inflammation of joints. The term rheumatism was probably introduced by Galen in medieval times when at that time pain was thought to be caused by one of the four cardinal "humours" (L. liquids).

Although rheumatic conditions are among the most ancient of human diseases, paradoxically rheumatology is one of the most modern branches of medicine. By 1953 it became a speciality in its own right and includes the study of diseases such as rheumatic fever, gout, osteoarthritis (OA), rheumatoid arthritis (RA), soft tissue rheumatism, spondarthritides including ankylosing spondylitis (AS), psoriatic arthritis (PSA), Reiter's disease and enteropathic arthritis and connective tissue diseases including systemic lupus erythematosus (SLE), systemic sclerosis (SS) and mixed connective tissue diseases (MCTD).

In this project I have been specifically interested in rheumatoid arthritis, using AS, PSA and SLE patients for comparative purposes only.

RHEUMATOID ARTHRITIS

Archeological evidence for RA is scanty and this, coupled with the absence of convincing early descriptions has raised the possibility that the disease may be one of fairly recent times, being scarce before the 18th century.

Robert Adams (1791-1875) can reasonably be regarded as the pioneer in rheumatoid pathology. Landre-Beauvais is usually credited with the first clear differentiation of the disease, which he called "goutte asthenique primitive" in his doctoral thesis of 1800. Alfred Garrod proposed the name "Rheumatoid arthritis" in the 19th century to replace many alternatives such as rheumatic gout, chronic rheumatic arthritis, and his son Sir Archibald Garrod continued this work into the 20th century. Jean-Martin Charcot (1825-1893) left drawings of the clinical deformities of the hands in RA, but stubbornly classified all forms of chronic arthritis as "Rheumatisme articulaire" and so held up progress in the field.

We now use the term "Rheumatoid Arthritis" (RA) to describe a "ubiquitous chronic inflammatory disorder of unknown aetiology which characteristically effects the synovial joints" [1]. Most doctors use the ARA criteria for its diagnosis (Table 1.1). It is a disease in which the control of inflammation and immunological processes seem to be aberrant. It has a worldwide distribution and in western communities is found in 1% of the adult population. It is more prevalent in females than men (3:1) [2], however after the menopause

Table 1.1 American Rheumatism Association diagnostic criteria for rheumatoid arthritis. Diagnostic classes divided into: 'classic' rheumatoid arthritis: >7 criteria; 'definite' rheumatoid arthritis: 5 or 6 criteria; 'probable' rheumatoid arthritis: 3 or 4 criteria; 'possible' rheumatoid arthritis: 1 or 2 criteria.

<i>Criteria</i>
(1) Morning stiffness
(2) Joint pain on motion or tenderness
(3) Swelling of a joint
(4) Second joint involvement
(5) Symmetric joint involvement
(6) Subcutaneous nodules
(7) Positive rheumatoid factor test
(8) X-ray erosions
(9) Poor synovial fluid mucin precipitate
(10) Positive synovial biopsy
(11) Positive nodule biopsy

the ratio becomes 1:1 indicating possible hormonal influences which may be at play. This idea is further enhanced by the fact that women with RA often improve during pregnancy and worsen afterwards. The onset of the disease is 20 to 60, although it most frequently appears in the age group 35-45, so it is a young to middle age disease, but even children can present with a severe form of RA termed "Juvenile Rheumatoid Arthritis" (JRA).

The onset of RA can be acute, insidious, or palindromic. Patients present with joint pain, joint stiffness (particularly in the early morning, described as "early morning stiffness" (EMS)), swelling and systemic signs in response to this inflammation which include low grade fever, weight loss and fatigue.

With acute onset, joint symptoms appear within a few days or weeks, affecting many joints in a symmetrical fashion. Most patients, however, have an insidious onset where symptoms emerge over several months, gradually involving more joints in a roughly bilateral

distribution. With palindromic onset, symptoms flit between joints, lasting for only a few hours or days each time over a period of several weeks or months before becoming persistent in nature. The prognosis is worse if the patient is young (at the time of onset), female and seropositive for IgM rheumatoid factor (RF, see later), or if there is multisystem involvement, compared to if the patient is elderly (at the time of onset), male and seronegative for IgM RF or in whom involvement is restricted to the musculoskeletal system. The prognosis is also better if there is rapid onset than if it starts insidiously.

Except with severe multisystem involvement, it probably does not reduce longevity but it certainly reduces the quality of life and requires stoicism, common sense, patience and optimism from the patient. In the words of one sufferer, Dorothy Eden (novelist), "the most profound thing anyone ever told me about RA was that it wouldn't kill me, but as like as not it would make me wish I was dead. Instead of flying through life on the wave of achievement I've spent my most productive years watching my body disintegrate into useless twists and gnarls, and I have grovelled in frustration".

10% of people show short lived polyarthrititis followed by sustained remission, 10% show relentlessly progressive polyarthrititis and over 80% show relapsing and remitting additive arthritis. 50% become significantly disabled 10 years after onset [3]. Fortunately, with modern management techniques, the disease can be controlled in the majority of patients and only less than 10% become completely disabled.

THE MUSCULOSKELETAL MANIFESTATIONS OF RA

These are the signs that help the physician to classify an individual as having RA, since they are very distinctive to RA, which, unlike other rheumatic disorders, shows "symmetrical inflammatory polyarticular involvement".

The synovial joints involved are in the hands (PIP, MCP but rarely DIP joints), wrists, elbows, shoulders, cervical and upper spine, hips, knees, ankles, feet (MTP but rarely IP joints) and jaw, so virtually any synovial joint in the body, regardless of its size, can be affected.

Usually the first joints to be affected are the small joints of the hands, wrists, feet and knees, but as the disease progresses, larger joints become involved, including the elbows, shoulders, hips, ankles, subtalar and sternoclavicular joints and upper cervical regions of the spine.

In active disease the joints are inflamed, painful and each can be classified according to the degree of each of the five cardinal signs of inflammation, i.e. tumor (swelling), rubor (redness), calor (increased skin temperature), dolor (pain), loss of function as well as deformity, tenderness, abnormal range of movement, instability and crepitus. The articular inflammation may go into remission, as stated, but generally continues to cause "progressive joint destruction and deformity leading to various degrees of disability".

Radiologically RA patients show characteristic radiographic changes - there is evidence of loss of joint space and marginal erosions after persistent synovitis. Such changes are preventable but not reversible by second line antirheumatic drugs (see later).

Although a disease affecting synovial joints the systemic nature of RA is emphasized by the constitutional symptoms seen in the disease as described below.

EXTRAARTICULAR (EA) MANIFESTATIONS OF RA

75% of RA patients are believed to exhibit one or more manifestations of EA disease [4].

These include general constitutional symptoms of malaise, weight loss, fever and lassitude [5]. Some degree of anaemia is found in almost all RA patients, a finding common to most chronic inflammatory conditions [6]. In addition, EA manifestations can occur involving a number of other tissues of the body summarized in Table 1.2. The appearance of such features generally occurs in seropositive patients (see later). Of these the most common manifestation is subcutaneous nodules, which appear at some time in about 20 to 25% of patients who are almost invariably seropositive. The nodules are firm masses, varying in size and shape within subcutaneous or deeper connective tissues. Histologically the rheumatoid nodule is composed of a central area of necrosis surrounded by a palisade of epithelioid cells with a variable degree of lymphocyte and plasma cell infiltration and vasculitis. The nodules may appear, disappear and reappear at any

TABLE 1.2 Extra-articular manifestations of RA

Complication	Comments
Vasculitis	Bland endarteritis; necrotizing arteritis, serious; rheumatoid nodules
Heart	Mainly pericarditis
Lung	Pleurisy; fibrosis
Ischaemic neuropathy	Symmetrical sensory, mild; patchy motor and sensory, serious.
Eye	Dryness (Sjogren's syndrome), common; scleritis rare, ominous
Lymphadenopathy	Not rare; local or general
Spleen	Rare; with leucopenia = Felty's syndrome
Kidney	Mainly amyloid, or drug induced
Muscle	Muscular wasting

time. Although they rarely cause problems, they can break down and become infected. Unlike the synovial membrane of RA patients, where there are lots of HLA-DR⁺ T cells (mostly CD4⁺), in a nodule there are fewer lymphocytes, the T cell CD4:CD8 ratio is normal and HLA-DR expression is mainly on macrophages.

As stated, a wide variety of vasculitic lesions often occur in RA, generally in those patients with longstanding disease and high rheumatoid factor titres and nodules; in fact, vasculitis is thought to be responsible for many of the other manifestations of EA disease. The possibility that the vascular injuries are the result of oxygen-free radicals damaging important sulphhydryl-containing compounds needed for vascular integrity and blood flow is substantiated from fields other than rheumatology, e.g. gastroenterology, where it is common knowledge that sulphhydryl drugs afford cytoprotection to the gastric mucosa. Most of vasculitic lesions are clinically benign, such as those affecting the digital blood vessels characterized pathologically by intimal proliferation called bland endarteritis. Rarely, some patients (1.85%) develop a vicious necrotising arteritis called malignant arthritis causing necrosis of the skin, digital gangrene, neuropathic lesions and visceral infarction which may lead to death. Fortunately, this explosive arteritis occurs very infrequently. One or more of these EA manifestations can appear insidiously without being necessarily life threatening. The pathogenesis of rheumatoid vasculitis is unclear, though it is thought that deposition of circulating immune-complexes (ICs, see later) may play an important role. However, the exact relationship to vascular

damage has yet to be fully understood.

Vascular problems tend to occur either in longstanding RA, when very often there is no active synovitis because the joints have been damaged beyond repair - so called "burnt-out RA" - or abruptly at any time and simultaneously synovial and articular disease becomes clinically inactive. Therefore in this thesis such patients were avoided and only patients showing active synovitis were used.

A condition called Felty's Syndrome occurs in 1% of RA patients [1]. This variant of chronic RA was first described in 1924 and represents a very severe form of rheumatoid disease which is associated with splenomegaly and neutropenia [7]. Skin hyperpigmentation, leg ulcers, anaemia, thrombocytopenia and generalised lymphadenopathy may also be accompanying features [8]. The arthritis is usually at an advanced stage with numerous joint deformities and frequently appears as a burnt-out disease, although can appear as severely active disease. High rheumatoid factor titres are often observed in these patients. It has been suggested that hypersplenism is the cause of the neutropenia, but in some patients the neutropenic abnormality does not respond to splenectomy.

Since RA is defined as a "symmetrically inflammatory polyarthritis involving predominantly the small joints but which may eventually effect almost all the synovial joints in the body", it is necessary to understand the pathological changes that occur in such joints. But first it is necessary to describe the structure of normal synovial joints.

THE NORMAL SYNOVIAL JOINT STRUCTURE

Within the human body there are two types of joints, called synovial joints and cartilaginous joints, but only the former are effected in RA. A synovial joint can be defined as "two articulating ends of bone covered by hyaline cartilage and enclosed in a capsule lined by fine synovial tissue enclosing a potential joint space". These can be further divided into those with an articular disc in the joint space (spinal synovial joints) or those with no articular disc in the joint space, called diarthrodial joints and most of these are peripheral. There are several types of synovial joints that can be classified according to the shape of their articulating surfaces which in turn determines the type and extent of joint motion. The seven types of articulating surfaces are illustrated in Fig. 1.1.

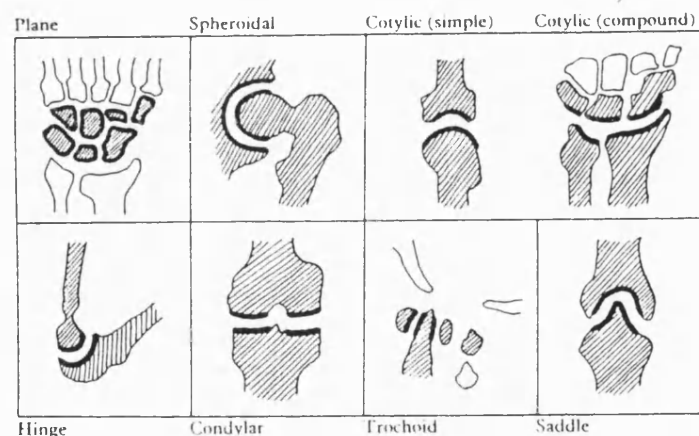


Fig.1.1. Summary of different types of synovial joint

Plane joints include carpal and tarsal joints of the wrist and the ankle respectively, spheroidal joints (also called ball and socket) include the joints in the shoulder and hip. Simple cotylic include MCP joints of fingers. Compound cotylic include radiocarpal joints of the

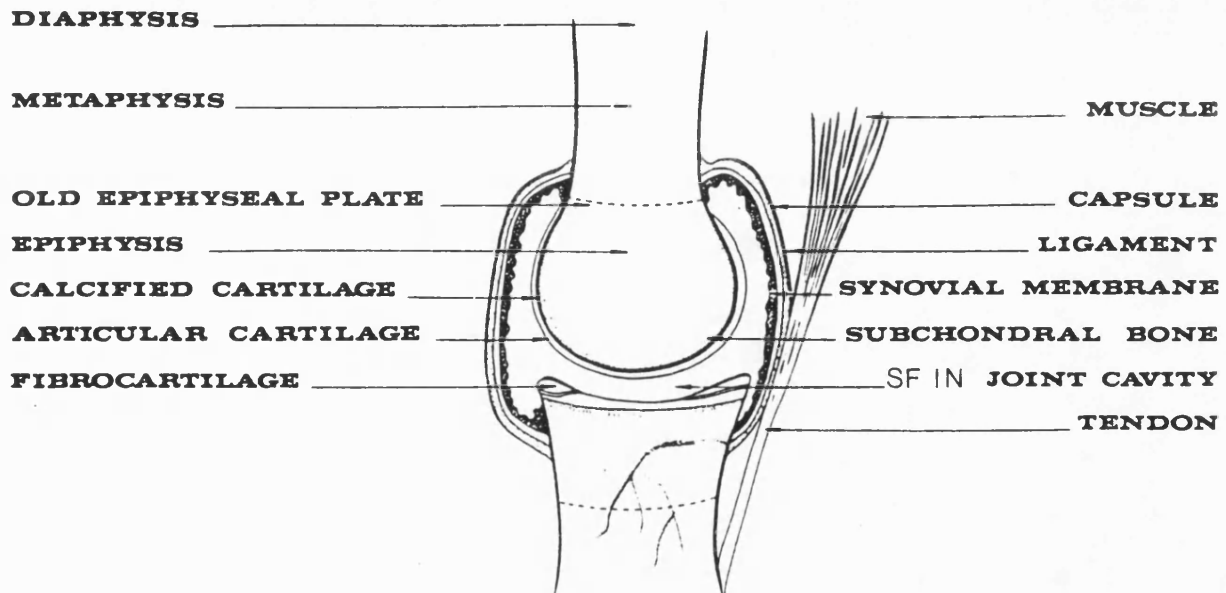
wrist; hinge joints, e.g. interphalangeal joints of the hand and feet and elbow joints; condylar joints, e.g. knee; trochoid (also called pivot) joints, e.g. proximal radio-ulnar joints; and finally saddle (also called sellar) joints, e.g. carpometacarpal joint of the thumb.

A diagrammatic representation of a typical synovial joint using a spheroidal joint to illustrate its features is shown in Fig. 1.2. In all cases the synovial joints' structure is as follows:

synovial joints possess a joint cavity which permits them to be freely movable. Such joints are encompassed by a capsule consisting of a dense fibrous outer layer (fibrosum) which diminishes towards the articular cavity giving way to a thin lining of synovial membrane (SM) also called the synovium. This membrane encapsulates the whole of the articular cavity except at those places where the ends of the opposing bones are covered by articular hyaline cartilage. The synovial cavity is filled with a highly viscous fluid called the synovial fluid (SF), which lubricates the joint surfaces.

The SM is considered to consist of two basic layers. The innermost layer lining the synovial cavity is known as the "intimal layer" and is predominantly cellular with an abundant blood supply. The intimal layer lies upon a less vascular subsynovial layer consisting of a meshwork of connective tissue. This can be mainly areolar, fibrous or fatty in its nature depending on its location in the joint. The outer part of this subsynovium merges with the fibrosum of the capsule. The basic functions of the SM are the production of the synovial fluid and the removal of debris and foreign material from the synovial cavity. Microscopic studies in the intimal layer distinguish two cell types,

FIGURE 1.2



Normal diarthrodial joint showing the relationship between the synovial membrane and the other principal anatomic components.

called synoviocytes [9]. The predominant cell type is termed type A, and is characterized by its large number of organelles including numerous lysosomes and vacuoles and small amounts of endoplasmic reticulum. These cells resemble macrophages in their characteristics, being active in phagocytosis and secretion and are involved in the removal of material from ^{the} joint space. In contrast, the type B cells have few organelles and possess abundant endoplasmic reticulum, and resemble fibroblasts. These cells are thought to produce the proteins and hyaluronate of the synovial fluid. In addition there are some cells that share the features of both major cell types; these intermediate cells have been termed type AB cells by Ghadially [10], who suggests that type A and B cells are not distinct races of cells but merely represent different functional phases of the same basic cell.

Synovial fluid is essentially a dialysate of plasma [11] to which 2% of hyaluronate has been added. This hyaluronate produced by type B cells of the synovium is a high molecular weight glycosaminoglycan responsible for the viscosity of the fluid and hence essential to its lubricating properties. The SF is normally clear, colourless and does not clot due to the absence of fibrinogen. It bathes the articular surfaces serving as a source of nutrient for the cartilage, it also carries material resulting from the daily wear and tear within the joint to type A cells for removal by phagocytosis. The SF contains very few cells and those that are present are thought to be derived from the SM lining the space.

The articular hyaline cartilage appears macroscopically as a

smooth glistening structure covering the ends of opposing bones and is the weight bearing surface of the joint. In adults it is an avascular, an alymphatic and aneural tissue, but is metabolically active and is composed of a small number of cells called chondrocytes which synthesise the other components of the cartilage structure, namely an abundant matrix and fibres. The cartilage derives nutrients from the SF by diffusion. The matrix consists of a framework of collagen fibrils (mainly collagen type II), which are composed of three coiled polypeptide chains arranged into a triple helix configuration. These molecules are quarter staggered to each other to form the fibrils which in turn form the fibres. The latter vary in thickness and distribution within the cartilage [8]. The collagen fibres entrap a ground substance rich in water and proteoglycans. The proteoglycans comprise repeating disaccharide subunits linked covalently to a protein core and contain mainly chondroitin sulphate A and C and keratan sulphate [8]. These are attached via a link protein to hyaluronate forming large aggregates which are trapped in the collagen network. The functions of articular cartilage depend on its resilience and load bearing properties. These are due to the osmotic pressure of water taken up by the proteoglycans (which are viscous and hydrophilic) causing the collagen network to swell, however it is retained and restrained by the network of collagen fibres thus giving cartilage its firmness and elasticity [10]. Since the chondrocytes have lost much of their ability to undergo mitotic division, regeneration of cartilage is limited after damage or destruction.

PATHOLOGY OF THE RHEUMATOID JOINT

Correct mechanical functioning of the joint depends on maintenance of normal joint architecture. In RA, the normal structure becomes altered thus causing a disturbance in its normal function. This leads to the disability so characteristic of the disease, the degree of which is largely determined by the extent of the joint damage.

1. Early active synovitis

It has been difficult to appraise the early lesions that occur in the rheumatoid joint since most patients do not present until the synovitis is quite well established. Also synovectomies are not common, particularly in early RA. Vascular changes are amongst the earliest documented events from work by Kulka et al. [12] and Schumacher [13] who analysed biopsies taken from patients with disease of only six weeks duration; these authors found an increase in blood flow to the joint together with some oedema of the subsynovium. Microvascular injury is evident as demonstrated by endothelial cell damage, gaps between the endothelial cells, thrombosis of small vessels and extravasation of erythrocytes. The synovial lining cells undergo a mild hyperplasia and in some cases there is a small degree of polymorphonuclear (PMN) and lymphocyte cell infiltration into the superficial synovium and perivascular locations, but no plasma cells are seen. There is some evidence of phagocytosis. The SF also contains small numbers of mononuclear cells (MNC) and PMN. However all changes

were focal so that depending on where the biopsy was taken normal or abnormal appearances were found.

2. Acute active leading to chronic active synovitis

Descriptions of established disease are abundant. Most authors agree on the following course of events [14-16]. As rheumatoid arthritis becomes established, the synovium undergoes gross histopathological changes. The following is an account of acute synovitis. There is substantial hypertrophy and hyperplasia of the synovial lining cells. The intimal layer, normally only 1-3 cells deep, can reach thicknesses of up to 10-20 cells. Thus, the synovial tissue can increase 100-1000 fold and eventually weigh up to 50 g [113]. Within the intimal layer there are now multinucleated giant cells which are probably derived from type A cells [9], the hyperplasia seems to involve all the different synovial cell types, but while Ghadially [10] asserts that there is a predominance of type AB and B cells, other workers maintain that many of the hyperplastic cells appear to be the macrophage-like type A cells [17,18]. More common, however, are the interdigitating cells which occur in significantly greater numbers in RA synovium compared to those from normal or non-RA inflammatory joints [19]... These dendritic cells, which have long branching processes, lack macrophage, lymphocyte or fibroblast characteristics, but are strongly HLA-DR⁺. Just beneath the lining there is a rich capillary network. Many of these blood vessels have fenestrations such as those observed in glomeruli. As well as the formation of new blood vessels, vascular lesions observed in early

disease are exaggerated. Thus, venous distension, capillary occlusion, arterial wall infiltration by neutrophils, areas of thrombosis and perivascular hemorrhage are regular findings in established disease.

The inflamed oedematous synovium protrudes into the joint cavity as slight villous projections extending towards the articular cartilage. Most typical of established RA is however the extensive mononuclear cell infiltration (lymphocytes and plasma cells) into the normally acellular subsynovial stroma. Sometimes these cells are collected into aggregates, or follicles, particularly around smaller blood vessels [20]. Although, true germinal centers are not frequently seen and when present usually occur in longstanding RA [14]. When the germinal centers are full of lymphocytes, lymphoblasts and plasma cells, they resemble lymphoid tissue. The dominant cell within such aggregates is the B lymphocyte and they can produce the immunoglobulins which can form immune complexes [17,19]. Three types of distributions of lymphoid cells may be observed:

- a) Lymphoid rich areas
- b) Plasma cell rich regions
- c) Transitional areas where macrophages, lymphocytes and plasma cells are intermixed [21].

T cells are found perivascularly and B cells are located in the centres of the lymphoid follicles [22].

In some patients the various lymphoid cells are scattered throughout the synovial tissue. The majority of mononuclear cells within the rheumatoid synovium, however, are T lymphocytes which account for 85% of the lymphocytes found there [17,23-25]. The T

lymphocytes can produce lymphokines locally. Immune complexes and T lymphocyte products attract phagocytes. Neutrophils are attracted to the immune complexes by chemotactic products of the complement cascade, and are activated along with the other phagocytes by the immune complexes and lymphokines when they arrive. But, they too activate lymphocytes (e.g. neutral proteases are mitogenic for B lymphocytes), and products of monocytes stimulate synovial cells. However few neutrophils are ever found in the SM, since they tend to migrate through into the SF. Depending on the stage of the synovitis, the SM can be described as a site of acute inflammation where Arthus-like reactions are occurring or a site of chronic inflammation, with areas showing delayed type immune reactions [26]:

Along with changes in cellular structure of the synovium there is a concomitant increase in the volume of the SF (originally 2 ml), which shows extensive cellular infiltration. In contrast to early disease, the majority of these cells (75-90%) are PMN cells with the remainder consisting largely of lymphocytes together with some monocytes, macrophages and synovial lining cells [27]. PMN numbers range from a few thousand to tens of thousands per mm^3 in severe active disease [15]. This high cellularity gives SF its turbid appearance macroscopically. The normal capacity of cartilage to rebound from a deforming force is lost in a rheumatoid joint due to the loss of proteoglycans from the cartilage matrix (seen as a loss of metachromatic staining). Although most of the proteoglycan depletion occurs in regions where the inflamed synovium has encroached as what is called "pannus" upon the cartilage surface, depletion is also seen

at sites well removed from the advancing margins of the synovial membrane. Proteoglycan loss is probably a result of enzymatic digestion of the core protein of the proteoglycan subunit by proteases [28]. Such proteases are found within the SF that bathes the cartilage (described in section on pathogenesis of RA).

Cartilage destruction appears to occur in waves, each followed by maturation of the granulation tissue. The term pannus describes a vascular, fibrous granulation tissue arising from the perichondrial synovial membrane, which clings tightly to the cartilage surface and from which it cannot be separated [27]. The pannus has often been referred to as "aggressive, invasive, proliferative and tumour-like". It is said to be tumour-like in that it is inflammatory, hyperplastic, invasive and made of connective tissue, blood cells and immune cells and destroys and replaces cartilage. The pannus contains hyperplastic mesenchymal cells and there is a monocyte/macrophage mediated elevation of synoviocyte plasminogen activator (a neutral protease) which can contribute to the tissue remodelling and cell migration to be found in the pannus. The latter process has been termed synoviocyte transformation and is similar to processes occurring in tumours. Synovial plasminogen activator activity from monocytes behaves like a tumor promoter to turn synovial cells into tumour-like cells.

Three types of pannus have been described following electron microscopic studies on the cartilage-pannus junction [29]:

Junction 1 - resembles the synovium and comprises an area of intense proliferation of small blood vessels surrounded by highly cellular

infiltrates penetrating deep into the cartilage along the blood vessels and there is evidence of degeneration around the cellular accumulations. This area is the one resulting in the major destruction and erosion of cartilage, resulting from destruction by enzymes released mainly from monocytes in the area.

Junction 2 - resembles granulation tissue and comprises phagocytic cells and fibroblastic cells invading the cartilage. Again, erosion here is due to enzymes released from the infiltrating cells.

Junction 3 - is the least destructive junction and is where fibrous pannus overlays the cartilage and where the pannus contains no blood vessels and no active cellular reaction. This area causes the least erosive damage, but any damage is the result of interference with cartilage nutrition by blocking the arrival of nutrients from the SF. This type of junction may replace cellular pannus in time. It is not known whether each kind of junction progresses to the next or whether they develop separately.

3. Bone destruction

When acute active synovitis persists for a long time it becomes called chronic active synovitis, and after many years of such chronic persistent RA there is characteristic erosion of subchondrial bone, first as a marginal or juxta-articular erosion, where the bone is not covered by the pannus, giving the articulating end a "chewed-out" appearance radiologically. Further destruction, and in fact the worst, is presumed to be caused by destruction by the pannus burrowing

through the disintegrating cartilage to the bone, but some bone loss may also result from increased bone resorption by osteoclasts. At this stage the condition is described as "burnt-out" disease, since there is very little or no synovium left and as such very little or no more synovitis.

The SF by this stage is greatly increased in volume and quite watery, so no longer able to provide adequate lubrication of articulating surfaces. This also results in a high intra-articular pressure, which may be instrumental in providing damage to the cartilage and bone; furthermore, it is believed to be responsible for extrusion of the joint cavity into the neighbouring soft tissues, causing much of the pain experienced by the patient upon movement. The rheumatoid synovium may produce factors that possibly contribute to the enhanced resorptive activity such as PGE_2 [30] and osteoclast activating factor [28]. The destructive changes to the cartilage and bone are the major features causing the disruption of joint function. Weakening of the joint capsule and tendons can occur along with ligament damage causing instability and subluxation (partial dislocation) and if severe can cause the complete elimination of joint space. Fibrosis (excess fibrous tissue) and ankylosis (stiffness and fixation of joints) of opposing articular surfaces can occur in severe joint damage leading to decreased freedom of movement, which is associated with the shrinking of the capsule and the increasing approximation of the ends of the opposing bones. All these changes lead to progressive loss of joint function and in turn are believed to be responsible for the musculoatrophy and contracture [31]. A

diagrammatic representation of the sequence pathological joint changes in a RA synovial joint is shown in Fig. 1.3. The patient is thus left highly disabled and in pain and probably by this time also has complications of EA disease described before.

PATHOGENESIS OF RA

RA, like all the rheumatic diseases, causes tissue damage/injury and therefore has an inflammatory component, but in RA this response dominates the picture and all cellular and soluble components in the joints interact in this response. Inflammation can be defined as the response of living tissues to a sublethal injury and its purpose is to restore the tissues to their normal function. In normal circumstances the inflammatory response must be regarded as a dynamic process designed to minimize local damage and inhibit its progression and is short lived (acute), but indolent (chronic) inflammation can occur as in RA. Chronic inflammation can occur if a) the body cannot rid itself of a harmful infecting agent, so a chronic immunological reaction occurs; b) inflammatory and immune immunoregulatory mechanisms break down in favour of maintenance of the inflammatory reaction, or even due to c) defective lymphocyte maturation.

The combined break down in regulation of the two major homeostatic mechanisms in the body, namely the immune system and the inflammatory system, leading to chronic inflammation and the characteristic dysregulation of the immune system result in the synovial joint damage and EAD manifestations of RA. In RA there is a persistence of both acute (dominated by vascular damage and PMN

Rheumatoid Arthritis

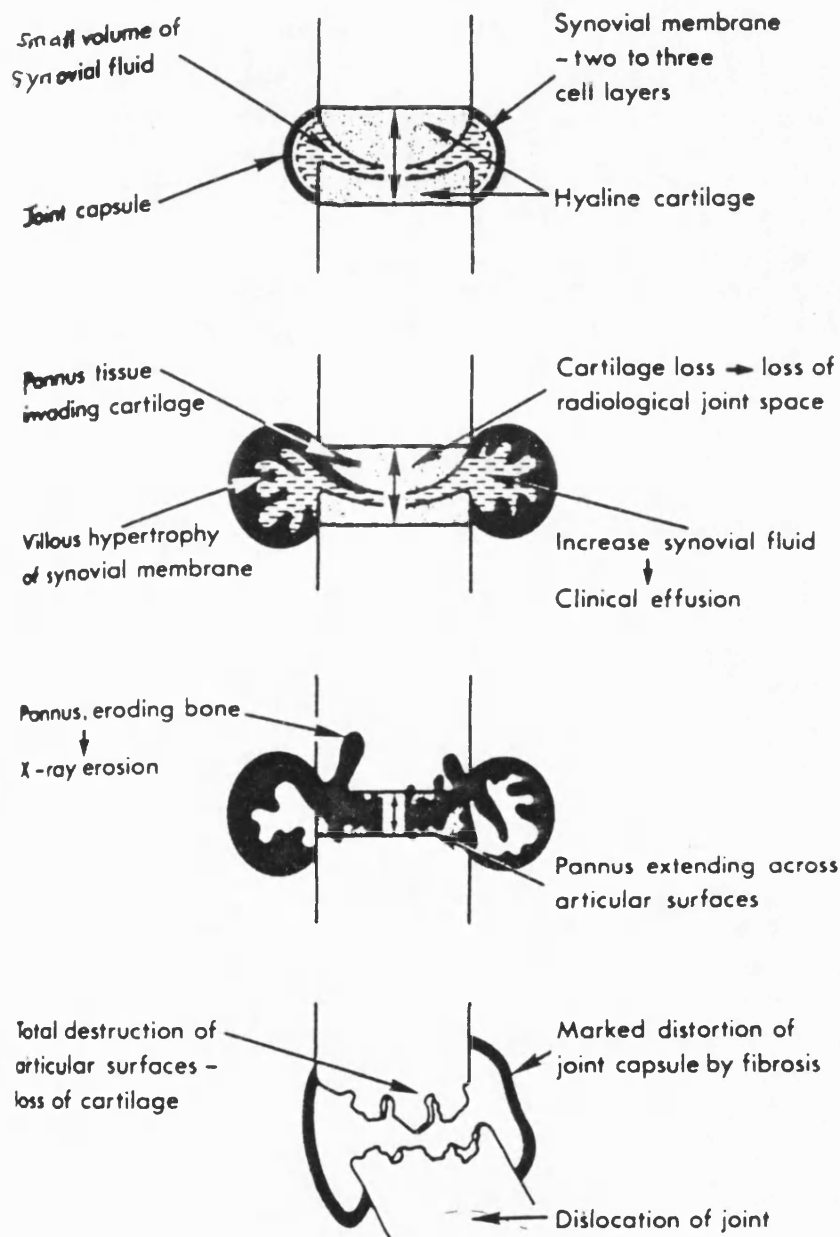


FIG. 1.3 Diagrammatic representation of pathological joint changes occurring during the course of rheumatoid arthritis.

infiltration and products from them) and chronic inflammation (dominated by MNC infiltration and the reparative phase and prominent immunological and systemic components).

Much of the damage seen in RA is immunologically mediated. This is evident not only from the joint pathology, where all manner of lymphoid cells and their products have been demonstrated, but also from in vitro and clinical studies, e.g. in vitro it has been reported that explants of rheumatoid synovia are capable of generating both antibodies and lymphokines [32], supporting the description of the rheumatoid synovia as an ectopic lymphoid organ [27]; clinical studies have shown that removal of circulating lymphocytes via the thoracic duct or by lymphocytapheresis produces symptomatic relief in patients with active disease, as can total lymphoid irradiation, whilst injecting these lymphoid cells back into the subjects exacerbates the disease process [33], suggesting that these processes remove pathogenic lymphocytes probably of B - or CD4⁺ lineage.

There is considerable evidence implicating both humoral and cell-mediated immunological processes in the pathogenesis of RA, with evidence of enhanced humoral immunity (raised serum Ig levels, evidence of rheumatoid factor and ICs and autoantibodies) and defective cell mediated immunity (abnormal number or function of T-lymphocytes). The rheumatoid joints show substantial infiltration by lymphoid and phagocytic cells and interactions between these cells and their products are thought to contribute to the tissue destruction so characteristic of RA. Within the synovia, lymphocytes, plasma-cells and macrophages are found in both diffuse and nodular patterns, an

arrangement that has been likened to an antigenically stimulated lymphnode [34].

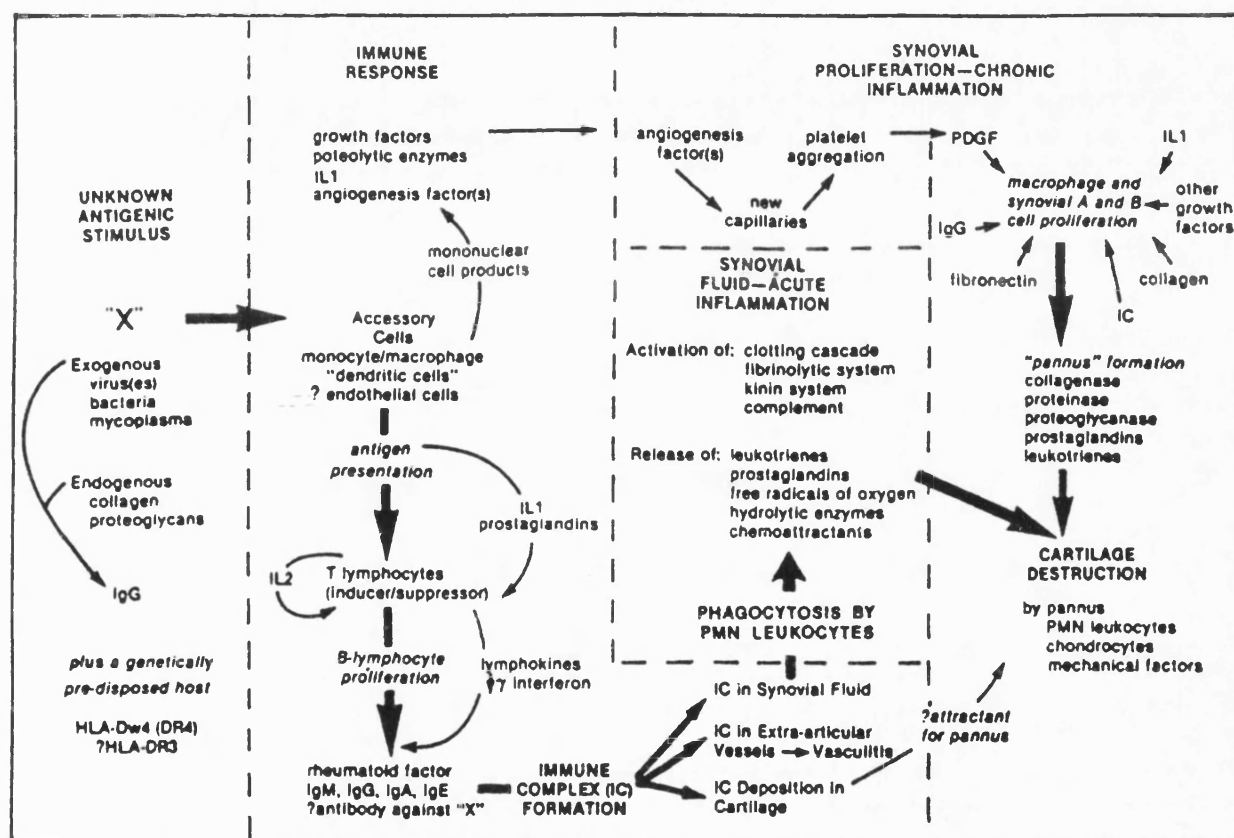
Table 1.3 summarizes the sequence of events which is currently thought to be responsible for the various pathological changes within the joints and which ultimately lead to its destruction and is discussed more fully below.

1. Phagocytic activity

The main destructive and inflammatory mediators in the synovial joint are products of PMN and macrophages, and interdigitating cells, namely lysosomal enzymes and reactive oxygen species which together with aminoacid metabolites are also able to promote and therefore perpetuate the inflammatory reaction.

Phagocytic cells are lured to the site of inflammation by chemotactic factors. Here their entry is facilitated by anaphylotoxins which enhance the vascular permeability, and also cause vasodilation, thus increasing the number of cells gaining entry to the tissues. Most of the phagocytic effector molecules are generated during the process of complement fixation and arise mainly from the cleavage of complement (C') components C3 and C5, thus C3a and C5a behave as anaphylotoxins. These, through direct action (C5a) or indirectly through mast-cell activation (C3a, C5a) - which causes release of the vasoactive amines histamine and serotonin - bring about the vascular changes described previously. C3a, and more importantly C5a, are also cast in the role of PMN chemotactants, so help promote influx of

TABLE 1.3 Pathogenesis of RA *



* from Harris Jr (1985) [15]

LEGEND TO TABLE 1.3

It is currently believed that the initiating antigen may become localised in the articular cavity, where local macrophage/dendritic cells process and present the antigen to T cells. There is release of IL-1 from the macrophages, activating a subpopulation of T cells to generate IL-2. This lymphokine produces proliferation and differentiation of other T cell populations. Thus inducer T cells are generated which through the medium of helper factors stimulate B cells to immunoglobulin synthesis. Immune complexes are formed and complement fixed, producing a number of anaphylactic and chemotactic factors. These increase the local blood flow and vascular permeability, leading to an influx of PMN and large molecular weight proteins such as fibrinogen and kininogen. The latter are activated, generating factors which further enhance the inflammatory process. Phagocytosis by both micro and macrophages results not only in the extracellular release of lysosomal enzymes capable of digesting articular tissues, but also in the production of pro-inflammatory arachidonic acid metabolites and oxygen free radicals. These, together with cell activation products, set up a series of highly complex, inter-related positive feedback networks which lead ultimately to the destruction of the joint.

PMN-cells to SM. C5a is a potent substance able to increase PMN adhesiveness to the endothelium of blood vessels, as well as incite these cells to oxidative metabolism, lysosomal enzyme release and the generation of aminoacid metabolites.

Other chemotactants include those derived from mast-cells, and the activated complex C5b67, the latter is also involved in "bystander attack" [35]. Although most of this complex is membrane-bound, some may be found free in the interstitial fluid. Here it binds to "innocent cells" in the vicinity and completes the C' cascade by fixing the remaining C8 and C9 components of complement, this results in cell lysis due to osmotic shock. It is believed that many of the vasculitic lesions produced within the rheumatoid joints and elsewhere in the body are due to the deposition of ICs in the blood vessels, which then bind C' with all its attendant effects. RA has in fact been described as an "extravascular immune-complex disease" [8].

Both the alternative and classical pathways of C' are activated in RA, and ICs are major mediators of this effect. This activation is indicated by the significantly lower concentrations of C4, C3 and C2 and factors B and P in RA effusions, compared to those obtained from subjects with non-RA diseases [36,37]. This decrease is greater in seropositive disease, probably due to a more aggressive disease. Reduced SF C' levels correlate inversely with IC levels [38], suggesting high local consumption of C' by ICs. Serum C' levels are however present in normal levels or increased [39].

Lysosomal enzymes are released from PMN and macrophages either during the process of phagocytosis or following cell death. They contain elements which are able to degrade both components of cartilage, i.e. the proteoglycans and the collagen framework. The earliest cartilage damage observed is the loss of ground substance (proteoglycans). This is seen histologically as an absence of metachromatic staining. The aggregates of proteoglycan are broken down by the PMN serine neutral proteases elastase and cathepsin G, which act on the protein core and the link protein. The soluble components are then degraded intracellularly by the acidic cathepsin B and D. RA patients are also found to possess high levels of enzymes which are capable of digesting the polysaccharide side chains of chondroitin sulphate and keratan sulphate [15]. Once the proteoglycan is removed, not only is the cartilage less able to resist a deforming force, and therefore is at a greater risk of mechanical disruption, but its infrastructure is now open to proteolytic attack [8]. Elastase and cathepsin G solubilise the collagen by attacking the non-helical region of the molecule and breaking down the interchain cross linkage. At 37 °C the polypeptide chains unwind and are now susceptible to proteolytic attack by specific collagenase. This enzyme is elaborated by both the macrophages and the dendritic cells of pannus [40] and by the chondrocytes [41] in response to interleukin-1 (IL-1) produced by cells of the monocyte/macrophage lineage in the pannus [40]. So collagen break down occurs from above as well as from within the cartilage. Collagenase cleaves the polypeptide chains into two fragments which are further degraded to gelatin by a variety of

neutral proteases including specific "gelatinases" [15]. In severe aggressive synovitis, fibrils may be phagocytised whole by activated macrophages. Although protease inhibitors α_2 -macroglobulin and α_1 -antitrypsin are present within the joint, it is probable that these are saturated since free collagenase has been demonstrated in RA effusions [15]. Although this enzyme is released in an inactive form as procollagenase [42], the SF contains factors which are able to activate it. These include proteolytic enzymes and plasmin. Plasmin is generated from plasminogen in SF by plasminogen activator secreted by rheumatoid synovial cells [42], as well as the endothelial cells of small blood vessels in the pannus. Reactive oxygen species further enhance proteolytic attack by inactivating protein enzyme inhibitors [43].

Phagocytic cells (polymorphonuclear leukocytes, monocytes and macrophages) respond to a variety of membrane stimulants by the production and extracellular release of a variety of oxygen reduction products. This coordinated sequence of events is called the "oxidative or respirative burst". The respirative burst can occur following phagocytic activation (engulfment of bacteria or immune complexes) or simply by perturbation of the cell membrane by complement activation, RF stimulation or immune complex attachment. A variety of reactive oxygen species (ROS) also called oxy-radicals or oxygen-free radicals (OFR) are released including superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (OH^{\bullet}), hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2), although strictly speaking only $O_2^{\bullet -}$ and OH^{\bullet} are true radicals. Most are released directly into phagolysosomes but some are released

extracellularly. The respirative burst is initiated by an increase in oxygen uptake with consequent stimulation of the hexose monophosphate shunt pathway, followed by a one electron reduction of oxygen to its $O_2^{\bullet-}$ using NADPH or NADH as the electron donors catalyzed by NAD(P)H oxidase. $O_2^{\bullet-}$ is subsequently converted to H_2O_2 spontaneously or by enzyme mediated dismutation. In the presence of ferrous iron, H_2O_2 acts as a substrate, together with $O_2^{\bullet-}$, for the generation of OH^{\bullet} & 1O_2 called the Haber-Weiss reaction. ROS are also produced during oxygenase and oxidoreductase reactions, leaky electron transport chains and auto-oxidation reactions.

Normal quantites of ROS are physiologically important, functioning as bactericidal, tumoricidal agents and regulatory agents for T and B lymphocyte function, but excess ROS production has been implicated as a likely mediator of tissue damage in chronic inflammation. Some of their actions are listed below, including direct destructive action and promotion of inflammatory reactions:

- 1) Damage to endothelial cells in vitro [44,45] and reperfusion injury in vivo [46].
- 2) Connective tissue damage by degradation of macromolecules [47].
- 3a) Further enhancement of proteolytic attack of cartilage (proteoglycans and collagen framework) [48].
- 3b) OH^{\bullet} mediated depolymerization of hyaluronic acid [49] leading to the reduced SF viscosity seen in vivo.
- 4) Haemolytic anaemia [50] due to red cells lysis and subsequent release of free Fe which itself can stimulate ROS production.
- 5) Lipid peroxidation resulting in membrane damage mediated by OH^{\bullet} and

$^1\text{O}_2$ [50,51]. Polyunsaturated fatty acids being particularly susceptible to oxidation leading to the formation of organic radicals and lipid peroxides which fragment to produce gaseous hydrocarbons and low molecular weight reactive aldehydes and alkenes potentially able to further damage membranes and enzymes and possibly even cause cell death. This is a particularly dangerous process since it is auto-catalytic.

- 6) Inhibition of serum antiprotease activity [43,52].
- 7) Stimulation of lysosomal enzyme release, thus perpetuating inflammation.
- 8) Cleavage of covalent bonds in carbohydrates and proteins.
- 9) Inhibition of thiol containing proteins and enzymes.
- 10) Stimulation of the formation of chemotactic factors encouraging further phagocytic cells to the inflammatory site thus perpetuating inflammation [53-56].
- 11) Formation of clastogenic factors.
- 12) Auto-oxidative damage: a) to IgG causing antigenic sites to become available resulting in auto antibody formation [57], b) to collagen II (IV and VI) allowing anticollagen antibody formation [58] and c) to DNA causing anti-DNA antibody formation.
- 13) ROS can cause IgG to aggregate [59] and this can then cross-link FC receptors and stimulate neutrophils to produce more ROS and lysosomal enzymes [60].
- 14) Through non specific cell damage, ROS can activate the membrane enzyme phospholipase A_2 . This enzyme is found in a variety of cell types and along with ROS can be activated by a wide range of stimuli, including bacterial cell products, immune complexes and chemotactic

peptides. Its activation leads to the release of fatty acids from the plasma membrane of which arachidonic acid (AA) is the most important. AA can be metabolised by two enzymatic pathways generating two main classes of inflammatory mediators - the prostaglandins (PG's) and the leukotrienes (LT's). PG's are generated via the cyclo-oxygenase pathway, where AA is converted first to PGG_2 and then PGH_2 . From the latter are produced stable prostaglandins PGE_2 , PGI_2 and D_2 [61]. In platelets PGH_2 is converted to thromboxane and in vascular endothelial cells to prostacyclin; these mediators cause vasodilation and potentiate the pain produced by other inflammatory agents such as bradykinin and histamine [62]. PGE_2 is also known to promote bone resorption [30] and PG's, particularly of the E series, have been shown to possess immunoregulatory properties [63] (discussed later). The lipoxygenase pathway gives rise to leukotrienes arising from the conversion of AA to 5HPETE which can be processed to 5HETE or to the leukotriene LTA_4 . LTA_4 can be converted to LTB_4 or to LTC_4 , LTD_4 or LTE_4 ; the latter three together forming what is known as the "slow reacting substance of anaphylaxis (SRS-A)". Not only is LTB_4 a powerful chemotactic factor for PMN [64], but it also prolongs the increased vascular permeability produced by other mediators (e.g. C5a). LTB_4 has also been found to stimulate ROS production [65] and raised levels of LTB_4 have been reported in synovial joints [66].

15) Many stimuli that induce $\text{O}_2^{\bullet -}$ production also cause the release of lysosomal myeloperoxidase (MPO). This haemoprotein catalyses the halogenation of H_2O_2 into potent bactericidal agents (e.g. hypochlorous acid) [67]; MPO thus has catalase and peroxidase activity and its products can oxidise free amino groups to form chloramines

which are then able to oxidise free sulphhydryl groups. It can also generate cytotoxic aldehydes further able to damage cell membranes [68].

16) ROS can inhibit the function of the phagocytic cells which produce them [69], however, paradoxically, the reaction is not self limiting due to the short half life of neutrophils leading to rapid repletion by new undamaged neutrophils entering inflammatory sites.

17) At a biochemical level the ROS can readily react with lipids, nucleic acids and proteins. Interaction with membrane lipids producing lipid peroxides in a autocatalytic event could alter the structural integrity of the membranes, resulting in increased membrane fluidity and inability to maintain ion gradients [70]. The breakdown products of the lipid peroxides in the form of aldehydes could inhibit important intracellular enzymes, e.g. G-6-Pase, adenylate cyclase and cytochrome P450 [71]. Lipid peroxides could also damage extracellular proteins and enzymes. ROS can affect nucleic acids and DNA by causing scission and DNA fragmentation. ROS can damage proteins by destabilization or enhancing enzymic proteolysis [72,73]. ROS can oxidase lipoproteins, e.g. serum protease inhibitors, making them non-functional and resulting in the presence of dangerously high levels of proteases [43,52]. Although all aminoacids are vulnerable to oxidative attack, tryptophan, histidine, cysteine and methionine are particularly susceptible [72]. After oxidation the primary, secondary or tertiary structure of proteins may alter [72] unveiling regions recognisable by intracellular proteases.

Of particular interest to this project is the susceptibility of

cysteine residues to oxidative attack, since they are the only aminoacids containing a free SH group, which is known to be an important moiety in the function of many proteins required for cell function.

Oxidation of SH groups in the protein components of lipoproteins or glycoproteins in cell membranes could lead to the loss of cell function, e.g. a) by preventing receptor translocation to the membrane surface or receptor expression, b) oxidation within glycoprotein receptors could result in altered conformation of the active site and inhibition of receptor-ligand interaction and therefore inhibition of receptor function, c) oxidation of proteins on or near G proteins associated with cell membrane integral receptor proteins could lead to inhibition of signal transduction mechanisms and therefore cell activation.

18) ROS could inhibit PG production via membrane lipid peroxidation [74] and thus inhibit suppressor cells sensitive to this agent for their action.

ROS also show direct actions on lymphocytes and could therefore influence local immune responses, this is reviewed with respect to H_2O_2 , whose action is probably mediated by OH^\bullet in aqueous solutions. Thus disturbed immunoreactivity of SF lymphocytes could be due to local ROS production [91].

a) Selective lymphotoxic effects of H_2O_2 ; in order of increasing sensitivity being:

- red cells, resting monocytes, resting neutrophils;
- B lymphocytes;

- $CD4^+ T_{H/I}$;
- $CD8^+ T_C$, $CD8^+ T_S$, $CD4^+ T_{S/I}$, NK [75-78].

High local levels of H_2O_2 at inflammatory sites could therefore alter T cell subset ratios in favour of $CD4^+ T_{H/I}$ which are known to show increased resistance to secondary oxidant exposure, thus H_2O_2 could initiate and maintain T cell subset imbalances in immunoregulatory circuits. The different sensitivities of lymphocytes to H_2O_2 are not explicable in terms of different levels of scavenger enzymes and proteins, which does explain the greater resistance in the former cells.

- b) Inhibition of E-rosette formation [77,79].
 - c) Inhibition of the autologous mixed lymphocyte reaction (AMLR) [80].
 - d) Inhibition of antibody dependent cytotoxicity (ADCC) [81]
 - e) Inhibition of NK activity [82].
 - f) Inhibition of antibody synthesis and PFC to T-dependent antigens [80,83].
 - g) Inhibition of B-lymphocyte colony forming response induced by staphylococcus A [84].
 - h) Inhibition of CON-A induced capping [80].
 - i) Inhibition of T-cell mitogenesis. The order of decreasing sensitivity being: CON-A > PHA > PWM [79,83,85-87].
- However low dose H_2O_2 can be mitogenic per se [86,87]. Under certain circumstances $O_2^{\bullet -}$ [87b] and OH^{\bullet} [88] can also be mitogenic.
- j) Regulation of IL-2 production depending on dose. $10\mu M$ H_2O_2 stimulating IL-2 production, $30-100 \mu M$ H_2O_2 inhibiting IL-2 production [89,90].

k) Inhibition of SF lymphocyte reactivity [91].

Aerobic cells protect themselves from the toxicity of O_2 and ROS by various detoxification mechanisms described below:

a) Removal of $O_2^{\bullet-}$ intracellularly by an Mn containing superoxide dismutase (SOD) in mitochondria and cytosolic Cu and Zn SOD and extracellularly by trace amounts of SOD.

b) Removal of H_2O_2 intracellularly by a haem containing catalase (CAT) in peroxisomes and glutathione peroxidase in the cytosol and mitochondria and extracellularly by low levels of CAT.

c) Since free Fe and Cu complexes serve as catalysts for ROS production, their levels are kept as low as possible by binding to proteins. Intracellularly Fe is bound to apoferritin and Cu to histidine, and extracellularly Fe is bound to ferritin or transferrin and Cu to caeruloplasmin (CP).

d) CP has ferroxidase activity preventing lipid peroxidation and OH^{\bullet} generation and possibly weak SOD activity; it is also an inhibitor of Cu catalysed peroxidation, but, paradoxically, since it has oxidase activity, it can itself result in oxidative reactions and in particular it can catalyse the oxidation of SH containing molecules.

e) Included within membranes are enzymes that can stop the chain reaction of lipid peroxidation, e.g. Se containing glutathione peroxidase and the glutathione cycle enzymes for replenishment of reduced glutathione, the enzyme's cosubstrate, or e.g. vitamin E (α -tocopherol).

f) Dietary sources of ROS scavengers, e.g. high SH containing diets, vitamin C and D.

g) The main extracellular ROS scavengers include membrane bound thiol-containing molecules on B and T lymphocytes (particularly on $CD4^+ T_{H/I}$) and resting monocytes, neutrophils and macrophages and soluble serum proteins, e.g. albumin and low molecular weight thiol containing non-protein molecules e.g. cysteine and reduced glutathione.

Thiol-containing enzymes and proteins are some of the most susceptible molecules to ROS damage; it can quickly be appreciated that intracellular thiol groups are far more protected from ROS damage than extracellular thiol groups. Since the quantities of antioxidant in the body are only sufficient for normal levels of generation of ROS, when the production exceeds detoxification capacity, particularly in situations where the detoxification mechanisms are deficient in the first place, ROS can quickly build up and being some of the most inflammatory agents known to man, cause tremendous tissue damage and immunodysfunction. In such a situation, extracellular thiol groups would be particularly vulnerable in view of the lower scavenging systems there in the first place.

The potential for oxidative damage in the RA joint is enhanced by a reduction or absence of intracellular scavenging enzymes such as SOD or CAT [92] and reduced glutathione peroxidase activity (due to reduced Se levels, [93]) and an increase in free catalytic Cu (due to reduced histidine levels, [94,95]) and by depressed extracellular ROS scavengers such as SH groups [96], resulting in increased ROS damage. CP levels are often raised as a result of acute phase protein synthesis and due to their dual function can have harmful or ameliorating effects, however the latter beneficial effect is often

reduced since its peroxidase capacity is often depressed due to poor Fe^{2+} oxidase activity, perhaps due to ROS attack of the enzyme itself [97].

Direct evidence for ROS involvement in RA pathogenesis is difficult to obtain, due to the rapid disappearance of these radicals from the joints, although increased ROS levels have been observed in SF using chemiluminescent techniques. Most evidence for their involvement is therefore indirect or comes from in vitro studies. The following is a review of the evidence for raised ROS levels in RA:

- 1) Raised spontaneous H_2O_2 and $\text{O}_2^{\bullet -}$ production from SF and peripheral blood from neutrophils and monocytes [96,98,99];
- 2) PMN and lymphocytes infiltration into the SF and SM being indirect evidence of an ongoing inflammatory reaction;
- 3) the fact that bovine SOD (Orgotein) reduces inflammation if injected into synovial joint cavities;
- 4) the presence of IC's, RF and C3b etc. in SF and SM known to be potent activators of neutrophils;
- 5) evidence of free radical oxidation and peroxidation products in RA SF and serum, but not in osteoarthritis, indicative of a local and systemic inflammatory response [100,100b]; the levels of these products correlate with the levels of Fe^{2+} salts and disease activity [101];
- 6) altered Fe metabolism leading to "anaemia of inflammation". Evidence of increased transferrin and ferritin in SF, but reduced capacity to bind Fe [102], increased free Fe in SF and SM due to microhaemolysis [15]. The fact desferroxamine inhibits lipid

peroxidation and OH^\bullet formation by chelating Fe resulting in suppression of inflammatory reactions in animal models [103]. The fact that dextran bound Fe when administrated to RA patients increases inflammation and peroxidation products [104];

- 7) low levels of ROS scavengers in SF and serum [92];
- 8) damaged connective tissue [47] and the presence of auto-antibodies [58];
- 9) altered lymphocyte responsiveness and numbers [91];
- 10) altered Cu metabolism with increased free Cu in the serum SF and intracellularly due to reduced histidine paralleling the increased CP levels [94,95]. Oral histidine causing clinical improvement in RA;
- 11) structural changes to IgG in RA samples [105] similar to damages observed in vitro due to ROS [106];
- 12) defective monocyte accessory function in RA-NSAID patients which seems to be due to cell surface thiol oxidation since it is correctable by sulhydate drugs [289].

It has been estimated that in an inflamed synovial joint during phagocytic ingestion the SF could contain between 6 to 612 μM H_2O_2 , but in RA, where there is evidence of reduced intra and extra-cellular scavengers, levels far in excess of 612 μM could be achieved and such a concentration would be lethal to cells in its immediate vicinity and influential but not cytotoxic to those further away [75].

Further evidence of enhanced phagocytic activity in RA, suggesting that ROS production may be high, is indicated by a) increased lysosomal enzymes in SF; b) a high frequency of low buoyant density neutrophils (indicative of in vivo activation) contaminating

PBMNC preparations after ficoll separation [107]; c) increased spontaneous and stimulated production of IL-1 by peripheral blood monocytes in cultures and the presence of IL-1 in SF and d) increased DR and FC receptor expression on phagocytic cells. However a few authors have found defective phagocytic activity in vitro, e.g. decreased chemotactic responses, impaired phagocytosis, impaired bacterial killing, reduced ROS production; however it could be proposed that these observations were artifactual due to either a lack of stimulating factors present in the culture medium which are normally present in blood and SF or simply reflect in vivo prior activation of cells which can't be further activated.

2. Humoral immunity

Originally workers were more interested in looking for disorders in the humoral side of the immune system in RA, because the consequences of this were more obvious. For example there seems to be hyperactivity of B-cell responses, particularly in early active acute and chronic active RA, (which diminishes in remissions) as evidenced by the presence of rheumatoid factor, IC's, autoantibodies, hypergammaglobulinemia and in vitro hyporesponsiveness of B-cells suggesting prior activation in vivo. Evidence for in vivo activation of B-cells is shown by in vitro studies showing spontaneous Ig production, spontaneous B-cell proliferation, reduced B-cell density, and in vivo by high serum B-cell activating factor levels.

Polyclonal hyperglobulinemia in RA serum is evidenced by raised IgG and IgM levels, however IgA may be normal, raised, or frequently

reduced. At the same time, at the major site of inflammation, namely the SM, large amounts of Ig's are generated. About 25% of the Ig found in the SF is synthesised by the lymphocyte infiltrates in the synovial pannus [108]. These studies however did not define the antigenic specificity of the antibody produced, some studies have however shown the presence of autoantibodies to collagen [58], suggesting collagen acted as an autoantigen and was involved in the initiation and perpetuation of the chronic inflammatory process. One study using synovial explants from RA patients previously immunised with tetanus, showed the production of only very small amounts of specific anti-tetanus antibodies, suggesting that the cells were already immunologically committed to a reaction against another antigen prior to tetanus challenge [109]. It has been shown that the proportion of Ig produced in the synovium are 48% IgG, 18% IgM and 34% IgA [110]. Interestingly the IgG produced by the SM showed a restricted electrophoretic mobility with considerable enrichment of the IgG3 subclass [111]. Others have found an increased incidence of light chains [111,112]. Such restricted responses might indicate a selective stimulation of the RA synovial lymphoid infiltrate [113]. The major humoral immune response in RA is the production of rheumatoid factors (RF). The presence of RF is termed seropositivity, and its absence seronegativity and patients can switch from one to the other. RF are autoantibodies directed against the Fc region of IgG molecules. Rheumatoid factors are found in the IgG, IgM and IgA classes [114]. Although IgG RF is the predominant class, because of the unique properties of IgM RF, only the latter tends to be reported by laboratories. Other autoantibodies that have been reported in RA

include autoantibodies to collagen, DNA, $CD8^+$ T lymphocytes and $CD4^+T_{S/I}$. Hence RA is regarded as an autoimmune disease.

The pathogenic importance of RF in RA remains controversial. Synthesis of RF undoubtedly does occur in RA SM, but whilst early studies indicated that about 60% of synovial IgG producing cells produced IgG RF [115], recent studies showed very few synovial plasma-cells synthesizing RF [110], possibly arguing against any significant pathogenic role of RF in articular inflammation.

One possible explanation for RF production is the alteration of autologous IgG by e.g. reactive oxygen species leading to enhanced immunogenicity [57,116].

Both IgM and IgG RF can fix complement [117,118] and recently an immunoregulatory role for RF's as anti-idiotypic Ab's directed against anti-bacterial peptidoglycan Ab's has been suggested [119].

In the serum IgM RF may react with IgG to give immune-complexes with a sedimentation coefficient of 22 S, which are probably cleared by the reticulo-endothelial system [120]. IgG RFs can self-associate and become intermediate size immune-complexes, the presence of which has been demonstrated in rheumatoid SM of both seropositive and seronegative patients with active disease [111].

Such immune-complexes probably play an important role as inflammatory stimuli for RA synovitis [111,121], and may also impair Ig negative feed-back inhibition, thus causing a perpetuation of immune response and inflammation [122].

Whatever the specificity of Ab produced by rheumatoid synovial cells, one of the main postulates concerning the pathogenesis of inflammation in RA is the formation of immune-complexes in the joints [27]. Since immune-complexes can cause activation of the complement cascade.

Immune-complexes have been demonstrated in the SF and in the menisci and the cartilage of RA patients using immunofluorescence techniques [123]. Entrapment in the cartilage may be due to the charge difference between the macromolecules of cartilage and Ig [15]. Analysis of ICs have shown that although some contain anti-nucleic, anti-collagen and anti-fibrinogen Ab, most of the ICs within the joint contain RF [8].

However, despite considerable evidence relating disorders of humoral immunity to RA, the question still remains whether B-lymphocytes really play an important role in tissue lesions, since:

- 1) it has been noted that RFs are not specific to RA as they can be found in the sera of patients with other rheumatic diseases (e.g. SLE), and other non-rheumatic diseases (e.g. bacterial endocarditis), as well and in normal individuals [124];
- 2) RF is not present in all RA patients, thus is not a prerequisite for RA;
- 3) if RF is transferred from an RA patient to a normal subject there is no effect;
- 4) congenitally agammaglobulinaemic boys show an increased evidence of RA in which humoral responses are presumably of no importance [125];
- 5) a chronic arthritis can be induced in bursectomized chickens [126].

Thus most investigators now believe that disorders in humoral immunity

are secondary to disorders in cell-mediated immunity. Although one report stated that bursectomized or thymectomized chickens can develop synovitis, but the disease is worse if both organs are removed, suggesting that the two arms of the adaptive immune system act in concert.

3. Cell-mediated immunity

An increasing number of observations suggest that cell-mediated immunity plays a more important role in the pathogenesis of RA.

In RA SM the predominant infiltrating cell is the T cell [24] whilst the SF and supernatants of rheumatoid synovial tissues show T cell derived lymphokine activity [32].

A number of studies have shown impaired T cell function in RA. Some patients show depressed cutaneous hypersensitivity to recall antigens [127] and several studies have shown significantly depressed in vitro lymphocyte activation to T cell dependent antigens (PHA, CON-A, PWM) with respect to normals [128-130]; however others have shown normal responses [131]. The discrepancies may be attributable to patient heterogeneity and/or differences in methodologies. Some workers have suggested that decreased responses are peculiar to particular subsets of patients, e.g. those characterised by the presence of erosive joint disease [132] and those that display anergic delayed hypersensitivity in vivo [133]. Recently it has been suggested that poor proliferative responses to soluble antigens are a consequence of defective cell surface signaling rather than a defect

in the T cell proliferative mechanism [134]. Most patients display defective PWM stimulated Ig production [135-137].

Some patients show defective AMLR and allogeneic MLR and ADCC; there are numerous reports of defective T cell and non-T cell suppressor activity (discussed later), clearly suggesting that immunoregulatory dysfunction may be a major factor in the initiation and/or perpetuation of the disease.

The hyporesponsiveness of rheumatoid mononuclear cells in vitro is somewhat paradoxical, in view of the apparent hyperactivity of the immune response in vivo: the best explanation to date is that preactivation of the cells in vivo renders them unable to be triggered further in vitro.

IMMUNOREGULATION

In a chronic disease such as RA, it is usually not clear whether defective immunoregulation is secondary to the disease process or actually serves to initiate it, or at least allow its establishment and perpetuation. Inflammation is usually a self limiting process, but it is clear from the sections above that the immune system in RA is unable to contain the various inflammatory processes occurring in the joint and elsewhere in the body. This is either because the immune system is unable to effectively eliminate the initiating antigen or to adequately regulate the normal immune response to the causative agent. In both situations chronic inflammation is produced. Furthermore, autoimmune phenomena are highly characteristic of RA, where some 70%

of patients are seropositive [8]. In normal subjects, response to self antigen is suppressed or eliminated by regulatory pathways.

In view of the above it is probable that RA has a multifactorial aetiology which in subjects with defective immunoregulation leads to the development of a common pathway of inflammation which we term "Rheumatoid disease". This concept has led to the examination of immunoregulatory pathways in RA.

This section will be considered under the following headings, attention being drawn to 7a), 7b) and 8) of most relevance to this project.

Immunoregulation by:

- 1) Antigen itself
- 2a) Immunoglobulin
- 2b) Anti-idiotypic antibodies
- 3) NK cells
- 4) Veto cells
- 5) Prostaglandins
- 6) Non-T PBMNC - phenotypic and functional characterization.
- 7) T cells
 - a) Phenotypic characterization of T cells
 - b) Functional characterization of T cells
- 8) Cytokine production

1) Antigen itself

Since the maintenance of a given immune response is ultimately

determined by the availability of the Ag, it could be that in RA there is an inability to rid the body of a pathogenic antigen (see later).

2a) Immunoglobulin

Ig molecules can both enhance or suppress humoral or cell mediated immunity accomplished by a variety of mechanisms including Ag clearance, more efficient Ag localisation, the generation of subpopulations of T_H or T_S cells or anti-idiotypic antibodies.

Of particular relevance to this project is the fact that Ig molecules can inhibit their own production and this has been called "Ab-mediated negative feedback suppression" [138]. The first evidence for this pathway came when it was shown that passive administration of Ab was able to suppress specifically the response to a given antigen [139]. The inhibition operated at two levels [140]. At high concentrations the Ab masked Ag determinants but at low concentrations suppression was mediated by the Fc portion of the Ab. There are two schools of thought concerning the mechanisms whereby Fc-mediated suppression takes place. One idea is that it results from interference between T-B cell cooperation [140,141]. The second idea advocates direct blockage of B cells and diversion to memory cells [142a]. In 1971 the same authors proposed the tripartite model of Fc-mediated feedback suppression [142b], where Ig in immune complexes bound to B cell antigen receptors (giving specificity) and Fc bound to the Fc receptor. Cross linking of the two receptors was thought to inactivate B cells. The conflict between the two schools of thought remains unresolved, but the two concepts may not be mutually exclusive.

Factors affecting this Ab-mediated feedback suppression include class or subclass of Ab and the immune status of the host. Enhancement of the immune response can occur with particulate and soluble Ag often occurring at low concentrations of Ab and involving its Fc portions. Some authors suggest that the relative concentration of Ag and Ab in an immune complex dictate whether suppression or enhancement occurs [138,139]. Apart from Igs, immune complexes can regulate immune responses by inhibiting B cell responses or augmenting B cell responses by encouraging antigen presentation. Soluble T cell products can regulate Ab-mediated feedback suppression in vitro, e.g. T cell replacing factor (TRF) can inhibit this suppression if added before immune complex addition by binding to B cell Fc receptors and thus blocking their subsequent interaction with Ab in the immune complex [143], e.g. Immunoglobulin binding factor (IBF) from T γ cells when incubated with immune complexes can enhance Ab-mediated feedback suppression by binding to the Fc portion of the IgG molecule and inserting itself into the B lymphocyte membranes [144], e.g. γ Interferon stimulates Fc α b receptor expression but inhibits Ab mediated feedback suppression [144b].

As well as its immunoregulatory role, Ab in the form of immune complexes or Fc fragments has been shown to induce human cell proliferation which again is affected by class/subclass variation [145]. More recently it has been reported that a subfragment of the Fc fragment is able to act as an adjuvant in the immune response [146]. In view of the important immunoregulatory role of Ab on the immune response it is hardly surprising that many immune cells express

receptors for the Fc portion of Ig; it is expressed on 80-95% of immature B cells, monocytes, macrophages, PMN, platelets, all active killer cells, some null cells and 30% of resting T cells. FcR's have been described for all five classes of Ig's, i.e. IgG, IgM, IgA, IgD, IgE. Although not on the bulk of resting T cells, activation of T cells increases the percentage of FcR⁺ cells and plasma cells lose their Fc receptors.

FcR⁺ cells are involved in Ab-mediated cell cytotoxicity (ADCC), type I hypersensitivity reactions, phagocytosis, immunoregulation, etc.

FcR⁺ expression has been found to be raised in RA [146-149], particularly if there is systemic disease [150], but others report no differences compared to normals [151]. However FcR⁺ function has been shown to be impaired in phagocytosis and in ADCC probably due to blocking of FcR's by immune complexes [152], or due to loss of FcR's following immune complex attachment [153]. B cells also participate in FcR-mediated suppression by producing a suppressive B cell factor (SBF) and this has been found to be low in RA in vitro [154].

Recently a subpopulation of MNC has been described carrying a receptor for Fc γ fragment of IgG (Fc γ is the IgG molecule minus the CH₃ region) [149]. Fc γ R⁺ cells are raised in RA [149] (agreeing with the observation of increased FcR⁺ cells in RA [146-150]), but not in OA or AS and such cells were found to be similar to "third population" cells, i.e. L or null cells, but different in other respects. A rise in Fc γ R⁺ cells is related to a rise in IgG synthesis, so such a rise

seems to reflect an attempt by the immune system to limit the production of IgG by participating in regulatory feedback suppression [155]. In view of the B cell hyperactivity and autoantibody production in RA, one could question just how receptive are RA cells to this suppression. RA show defective Fc γ -mediated feedback suppression of IgG produced in vitro [156], and it has been shown that the suppression was due to blocking of accessory^{cell} function by Fc γ fragments [157]. Fc γ R⁺ cells seem to be involved in the early stages of an immune response [157], supporting the hypothesis that Fc-mediated suppression results from interference between T and B cell cooperation [140,141].

Recent work suggests that Fc γ R-mediated feedback suppression is not impaired in early seropositive or palindromic RA disease (but is slightly in early seronegative disease) and develops with progressive disease [144b]. It seems that modulation of RF production by FcR ligands is complex and it is likely that immunoregulatory pathways other than nonspecific suppression by Fc γ or immune complexes, such as antiidiotypic antibodies or T suppressor cells may be more relevant to IgM RF control. Whether with progressive disease B cells become less responsive to FcR-mediated feedback suppression or whether the capacity of Fc γ R⁺ or FcR⁺ to suppress becomes impaired is not known. What is clear is that defective Fc γ fragment suppression shows a different time course in RA than defective T-suppressor function, which is frequently worse in early RA [245,258].

The fact that depressed serum thiol levels are present in RA and SH groups are important in FcR function [389] could perhaps go partly

to explain defective immunoregulation here, particularly in the light of data showing 2-ME enhances the suppressive effects and FacbR⁺ expression [144b]. Another possibility is that high natural levels of lipocortins may be inhibiting FcR function and this would affect both phagocytosis and immunoregulation.

2b) Anti-idiotypic antibodies

The idiotypic network theory is one of the classic hypotheses in the field of immunoregulation. In 1974, Jerne suggested that the immune system is regulated through a network of idiotypic - anti-idiotypic interactions [158]. This concept is based on the observation that Ab, as well as recognising Ig, can itself be perceived as an immunogen. This is due to the presence of "idiotypes" which are unique to the Ag binding site of that Ab and are usually located in the hypervariable regions of the Ig molecule. Idiotypes (ids) are thus to be found on surface Ig (i.e. Ag receptor) of B cells as well as on secreted Ig. They appear also to be present on T cell Ag receptors [159] and have been demonstrated on T_H and T_S subsets [160,161]. Thus idiotypic regulation can occur at both cellular and humoral levels.

An Ag elicits the production of Ab₁ which gives rise to Ab₂ (anti-idiotypic) and this then results in the formation of Ab₃ (anti-anti-idiotypic) and so on, establishing a network. Each Ab either upregulates or downregulates the activity of the preceding component. This immunoregulatory ability is reported to be dependent on the dose and isotype used, e.g low doses of anti-id can greatly enhance the

expression of the appropriate idiotypic in response to a given Ag, while higher doses produce suppression [162].

Early studies showed that whilst families of Ab_1 express similar but not identical Ag binding sites [163], they could all be bound by one anti-idiotypic antiserum. This suggested that this Ab_1 carried a "shared", "public" or "cross-reactive" idiotypic (CRI). This would also explain why Ab_3 is often found not to express the same antigen binding specificity as Ab_1 , although it may carry the same idiotypic as Ab_1 . The occurrence of CRI's is found to depend on the nature of the immunising Ag and the genetic make-up of the individual [163].

It is found that auto-antibodies frequently express CRI's. The restricted nature of idiotypes has been demonstrated on RF [164], since the majority of IgM anti-IgG Ab's carried the Wa idiotypic. More recently similarities between some IgG and RF's has been reported [165], which may be explained by the recent observation in mice that auto-antibodies utilise only a limited number of V_H gene families [166].

The presence of CRI's is particularly pertinent to autoimmunity since some authors believe that a loss of self tolerance may result from such antigenic determinants. Furthermore, there exists much evidence suggesting that responses where CRI's are prevalent are particularly susceptible to idiotypic regulation. These two aspects of idiotypic are discussed below.

i) Induction of autoimmunity

It has been suggested that an invading microorganism may elicit an Ab response, where the latter carries an idiotypic which is shared with autoreactive lymphocytes [167]. Induction of T_H specific for the idiotypic could then provide help for the autoantibody response.

An alternative possibility is that during a normal immune response, immature B cells are generated, some of which are autoreactive [163]. The induction of tolerance requires high affinity binding of immature B cells to Ag [168]; low affinity binding inhibits tolerance. The authors of this hypothesis suggest that Ab which crossreacts with (i.e. is anti-idiotypic for) Ag receptors on autoreactive B cells, does so with low affinity and therefore could inhibit the tolerisation of these B cells and thus lead to the production of autoimmune phenomena.

Clearly in both hypotheses a defect in the immunoregulatory T cell circuit is required for autoimmunity to arise.

Another mechanism whereby idiotypic may cause loss of self tolerance is through the occurrence of "internal image". Anti-idiotypic and idiotypic are mutually inducible, i.e. Ab_2 can induce Ab_1 . This occurs because Ab_2 is believed to carry the "internal image" of the Ag, which gives rise to the formation of Ab_1 . This idea has been used successfully in infectious diseases, where individuals can be vaccinated against Ag's which are highly infectious or not readily available, by using Ab_2 [159].

It has been suggested that autoimmunity may arise if the immune response generates anti-id Ab's, which carry the "internal image" of an autoantigen. Recently experiments were carried out using an anti-Ab which bore the "internal image" of human IgG Fc; this was raised using IgM RF from patients and was used to show that "internal image" anti-id Ab's could behave like Ag. Thus a proliferative (but not secretory) response was obtained from patients with RA but not from normal individuals. Furthermore the anti-id blocked RF synthesis by PBMNC but had no effect on the total IgM response following PWM stimulation [169].

Such idiotypes are therefore potentially powerful tools for analysing antigenic structures.

ii) Regulation of the autoimmune response

A number of investigators have reported the occurrence of anti-idiotypic Ab's in patient's sera coincident with clinical improvement, e.g. a) anti-id Ab's to autologous anti-DNA were observed in SLE patients and this was associated with decreased disease activity [170]; b) in RA in vitro suppression of monoclonal RF synthesis by the corresponding anti-id Ab has been reported [171] and in vivo decreased RF activity in the serum of a female patient with monoclonal IgM gammopathy following the appearance of an anti-id Ab has been reported [172].

Observations such as these, and the presence of CRI, clearly indicate an opportunity to manipulate autoimmune behaviour. Although

anti-idiotypic therapy has been used with some success in treating human B cell lymphoma, the use of such agents has not proved very fruitful in autoimmunity. This is because suppression of id^+ clones leads, after a short latency period, to the emergence of idiotype negative Ab's of the same specificity. Furthermore, side effects such as serum sickness and immunosuppression have to be considered.

3) Natural killer cells

Over the last decade practically all major cell types have been considered to be involved in immunoregulation to a greater or lesser degree. One recent contender is the NK cell. These cells are large granular lymphocytes which share some characteristic T-cell surface Ag's, e.g. CD8, and CD2, and can be detected by using a combination of Mabs, e.g. NKH-1 specific for CD56, NK-15 (Leu-11) specific for CD16 (Fc receptor of IgG), HNK-1 (Leu-7) CD57 and anti-CD11 Mab. They have been reported to modulate B cell differentiation [173] and Ig production [174,175], by production of IL-1, IL-2, BCGF and γ -IFN. There appears to be some dispute over the exact mechanism whereby NK cells mediate their suppressive effects on B cells, since T_H cells [173], antigen exposed accessory cells [175] and B cells [174] have all been described as the target cells. It is even possible that they can regulate IL-2 production.

Phenotypic marker studies show NK numbers to be normal in the blood, but their activity to be reduced, and in the RA synovium there appears to be a lack of NK numbers and cell activity [176,177], which is perhaps not surprising since NK function is highly dependent on

activation by IL-2 [178], whose production is defective in RA. In view of its effect on Ab synthesis, an absence of NK cell activity in an SM synovium already deficient in T_S function would serve to further enhance the B cell response.

4) Veto cells

Since veto cells are important in the maintenance of self tolerance and negative regulation of the ongoing immune response, it is possible that defects in their numbers and/or functions could have a role in the pathogenesis of RA.

5) Prostaglandins

Prostaglandins are a family of 20 carbon aliphatic ^{fatty acids} unsaturated, which are generated by every tissue in the body except for the erythrocytes [63]. They were originally described as smooth muscle stimulants [61], but have since been found to be potent immunoregulators, especially of cellular immunity and at physiologically relevant concentrations, apart from their well known action as inflammatory mediators. PG's have thus been shown to a) inhibit T cell mitogenesis in response to PHA and CON-A [179], b) inhibit E-rosette formation [180], c) inhibit T_c generation in MLR [181], d) inhibit the formation of T cell colonies in vitro [182] and e) inhibit γ -IFN production (which in turn is thought to be responsible for the continued growth of EBV transformed cells from RA patients rather than due to defects of T_S activity) [183].

A number of investigators believe that some of the above effects may be achieved through PG action on IL-2 synthesizing cells, since IL-2 production has been shown to be inhibited by PGE_2 in both animals [184-186] and humans [187,188].

Furthermore, PG's may also prevent cells from responding to IL-2, e.g. PGE_2 inhibited the proliferation of IL-2-dependent T cell lines in response to exogenous IL-2 [188].

Some authors believe that the inhibitory effect of PGE_2 on IL-2 production is through direct action [186,188], whilst others hypothesise that IL-2 production is suppressed indirectly through the induction of irradiation-sensitive, PGE_2 sensitive CD8^+ T cells [189]. It has been suggested that the conflicting reports on the absence or presence of such a T_S cell may depend on the concentration of PGE_2 used in the experiments [188].

PGE_2 has been reported to decrease NK and ADCC activity [190,191], which is interesting in view of a finding that PGE_1 increased FcR expression [192]. NK activity is diminished in RA and increased PGE_2 activity may be one of the contributory factors.

There are conflicting reports on the ability of PG's to regulate humoral responses, e.g. in man the proliferative response to PWM is not affected by physiological concentrations of PG [179,193,194] but that induced by staphylococcus aureus (SA) is suppressed, suggesting different B cell subsets may vary in their susceptibility to PG-mediated regulation. Likewise, the effects of PG's on antibody

responses has also produced conflicting data, e.g. one report showed PGE_2 could cause a dose-dependent inhibition of Ig secreting cells in PWM and SA stimulated B cell cultures (the PGE_2 suppressed the production of B cell differentiation factor (BCDF) from mitogen stimulated T cells and also the capacity of B cells to respond to BCDF) [195]; in contrast PG synthetase inhibitors have been found to suppress the Ab response of PBMNC in man, and this suppression is reversible with low concentrations of PGE_2 . However, the IgM response was not fully reconstituted by PGE_2 , suggesting other cyclooxygenase products may be more important in modulating this class of response [193].

It has been shown that PGE_2 does not affect B cells or monocytes directly [196], but probably the effects on B cells were mediated through action on T_H or T_S cells. The same author suggests that T_S cells, which inhibit Ig production, are themselves suppressed by PGE_2 and that NSAID inhibits PGE_2 production and releases the inhibitory influence on T_S cells which can then suppress an ongoing Ab response.

Support for the above hypothesis comes from the observation that T_S cells defined by $\text{Fc}\gamma\text{R}$ expression carried large numbers of PGE_2 receptors [197]. Most of these cells appeared to be CD8^+ , and removal of these cells decreased the amount of suppression of Ig synthesis observed with indomethacin, but did not reverse it completely. The lack of complete reversal has been suggested to be because some CD4^+ cells can be induced to become T_S cells [196] - without undergoing any phenotypic changes in the process -, by other CD4^+ cells [198]).

On the other hand PGE_2 has been shown to stimulate certain T and non-T suppressor cells that regulate IL-2 production [189,199,199a,273] and some PWM-induced CD11^+ T8 cells [200].

With reference to RA, where large quantities of PGE_2 have been reported to be present in the SF [201], it is clear that its presence may have profound effects on the immune function of the synovial tissue. Firstly it would explain the depressed T cell responses seen in the RA synovium; secondly it would provide an explanation for the exuberant Ab response in these tissues; thirdly the finding that cyclooxygenase inhibitors decrease IgM RF production in RA patients [202] would suggest that the high level of RF in RA joint may be due to the suppression of T_S cells by PGE_2 , which in normals suppress RF production [196].

Although it is believed that PGE_2 may mediate its effects on cellular and humoral responses by altering the intracellular cAMP and cGMP levels, it should be noted however that changes in the concentrations of these nucleotides and changes occurring in cell function do not always correlate [188,203]

6) Non-T PBMNC phenotypic and functional characterization

Enumeration of total B cells by Mabs or immunofluorescent labelling for surface Ig has shown relative numbers and percentages in RA blood to be within the normal range.

Differential blood counts reveal no gross differences in immunoregulatory cell numbers of lymphocytes or monocytes, although neutrophil contamination of PBMNC preparations accounts for the increased WBC count, indicating prior activation of neutrophils in vivo.

Higher numbers of $CD5^+$ B cells have been observed in RA [204] and are thought to be committed to autoantibody production [205]. There is also an increased number of activated B cells in the blood compared to normals [206] as indicated by a decreased number of mouse red blood cell rosetting cells [207], and this increased activation seems to correlate with the degree of active synovitis [208]. Even in normals there are always some activated B cells in the circulation, due to an ongoing immune response against daily encountered environmental pathogens. Decreased SBF has been found after HAGG (to mimic IC) stimulation of B cells and this correlates directly with aging and inversely with disease activity, suggesting that this could be one factor causing uncontrolled Ab and IC formation and help to account for the pathogenesis and chronicity of RA [154].

Monocytes, macrophages and B cells play a minor role in the suppression of immune responses, but defects in numbers or functions even at this level could be significant in the pathogenesis of RA.

7a) Phenotypic characterisation of T cells

T-cells are usually considered to be the main proponent of immunoregulation.

Two basic approaches can be used to study immunoregulatory T cells: one consists of phenotypic quantification of lymphocyte subsets with putative regulatory properties using Mabs specific to particular subsets; alternatively one can carry out functional analyses of the regulatory subsets.

Based on the fact that it is possible to detect T lymphocyte subpopulations with unique biological functions on the basis of their cell surface antigenic components using Mabs; Mabs have been widely used in various disease conditions including RA (on PBMNC, SF and SM cells) to provide immunodiagnostic clues.

Enumeration of total T cells by E-rosetting techniques and anti-CD3 Mabs has shown that their percentage and numbers in the blood are within the normal range, but there is an increased number of activated T cells and in synovial fluid [209].

As will be seen with respect to RA, phenotypic reports have been very conflicting, particularly in the blood, sometimes possibly because heterogeneous populations of RA patients in terms of disease activity and/or drug treatment were used and also because of the differing Mabs elected to be used by the different laboratories. The results seem to become more consistent as one moves from the blood to the SF and to the SM.

Most reports are based solely on comparisons of the ratios of $CD4^+$ to $CD8^+$ cells which were termed T helper (T_H) or T suppressor (T_S) cells respectively, but during the period 1971-89 ideas about the

functions of cells bearing such phenotypes have altered and it is now agreed that there exists enormous phenotypic and functional heterogeneity within each of these subsets, such that even the terminology $T_{H/I}$ (because some $CD4^+$ T-cells are inducers of various cell types including T_S cells as well as helpers for T-T, T-B and T-macrophages interactions) and $T_{S/C}$ (because some $CD8^+$ T-cells are cytotoxic as opposed to suppressor cells) are insufficient.

Using combinations of anti-CD4 or anti-CD8 Mabs with various new Mabs, functionally distinguishable subsets are continuously been found; with helper and suppressor activity even amongst both major subsets. Heterogeneity exists within each subpopulation with respect to their activation, growth and differentiation requirements and different modes of action and target cells. They also differ in radiation, steroid, PG and mytomicin C sensitivity, however a review of such subsets will not be given here.

Even to date, Mabs tend to be nonspecific and recognise antigens on other cells, so the long term aim is to find a battery of Mabs with better specificity that define functionally unique T cell subsets to provide a better correlation between phenotypic and functional aspects of T cells. It may well be found that such new antigens are important in the function of the cells in question, just as the CD4 and CD8 (and CDW29) antigens have proved to be important not just in cell recognition and adhesion, but also regulating TCR function. It would also need to be proved that marker-function relationships are the same in health and disease. Once all these improvements and proofs have been made, phenotypic analysis will be a more reliable prognostic and

immunodiagnostic technique than it is at present.

Of relevance to this project is the fact that the T4 subset includes the $CD4^+ 4B4^+$ memory cells ($T_{H/I}$) which always function in a helper capacity and induce other cell types (e.g. augment Ig production [210-213]) and $CD4^+ 2H4^+$ virgin cells ($T_{S/I}$) that function in an inducer capacity of $CD8^+$ suppressor cells [210,212-213]; the $CD8^+$ population includes $CD8^+ CD11^+ Tp44^-$ cells with suppressor properties for cell-mediated immunity and humoral immunity in the presence of monocytes and $CD8^+ CD11^- Tp44^+$ cells with cytotoxic properties. Most phenotypic analyses have therefore reported $CD4^+$ and $CD8^+$ and more recently $T_{H/I}$ and $T_{S/I}$ in terms of percentage of total PBMNC or T cells, or as ratios such as $CD4^+:CD8^+$ (normally 2:1) or $T_{H/I}:T_{S/I}$. Since percentages or ratios do not inform one of quantitative differences, wherever differential blood counts measuring total lymphocyte numbers were carried out, numbers of individuals subsets were calculated based on the percentages found.

i) Peripheral blood

Analysis of T cell subsets in RA peripheral blood has shown the percentage of T_H and $T_{S/C}$ cells to be within the normal range [209,214-216], whilst others have found the proportions of $CD4^+$ cells to be significantly increased and those of $CD8^+$ to be significantly reduced compared to normals [217-218]. This discrepancy is probably related to disease activity, since it has been reported that only patients with active disease have reduced percentages of $CD8^+$ cells, whilst those with inactive disease do not differ from normals

[219-220] In active disease a reduction in absolute numbers of $CD8^+$ cells has also been reported [216,218,220]. Since total T cell numbers seem unchanged, the difference may be made-up by "third population cells" which show increased EA-rosette formation [148].

Another possibility comes from the recent observation that there is a reduction in $T_{S/I}$ cells in RA and other articular diseases [221], which might explain the deficit of $CD8^+$ cells reported by some of the above authors, since $CD8^+$ cells are dependent on products of $T_{S/I}$ cells for their activation and function.

In vivo activation of peripheral blood T cells in RA has been reported to be normal [220,222] or increased [209,223,224] as indicated by the expression of class II antigens. The activated cells seem to belong predominantly in the $CD8^+$ population [209]. Using a decrease in lymphocyte density as an indicator for cell activation, it has been found that increased numbers of low density lymphocytes occurred only in patients with active synovitis, but not in those with EAD [208].

ii) Synovial fluid

Most authors agree that the percentage of $CD8^+$ cells is enhanced in the SF of RA patients [209,216,218,219], whilst that of $CD4^+$ cells may be normal [220] or reduced [209,216,218,219] in comparison to peripheral blood. The increased $CD8^+$ cells correlate with disease activity [225]. The increased $CD8^+$ cells in the SF could be due to a homing mechanism ($CD8^+$ cells have been demonstrated to show

preferential homing to sites of inflammation [226]) or may be due to selective proliferation in this region. The rise in Fe binding proteins in the joint could influence the migration of lymphocytes to the joint and their subsequent activation since lymphocytes possess Fe binding protein surface receptors [227].

These observations are surprising in view of the depressed levels of $CD4^+ 2H4^+$ cells in the SF [221]. It has been suggested that the increase in $CD8^+$ cells in the SF but their reduction in the peripheral blood is due to the migration to the SF where they become activated [216]. Although many of the cells in the SF appear activated [209,216,220,224,228], there is a discrepancy as to which subclass the majority of these activated cells belong. One report stated that class II antigen appeared on both $CD4^+$ and $CD8^+$ cells [220], but the majority of activated cells were $CD4^+$; this contrasts with other reports stating that the majority of activated cells were $CD8^+ T_{S/C}$ cells [209,216]. Another report suggested that $CD8^+$ population may reflect a subset of T8 cells [229] found within $DRW6^+$ and $DRW6^-$ Vicia Villosa adherent populations which have a dual function: that of Ag binding and presenting to T_H cells and to prevent suppressor cells from inhibiting helper cells, and so have been called contrasuppressor (CSC) cells [230]. This seems particularly likely in view of the fact that $CD8^+$ cells in the SF appear to augment Ig synthesis [231] and stimulate AMLR [232] rather than inhibiting them.

iii) Synovial membrane

Reports of the proportions of T cell subsets in the SM have been conflicting probably due to differing methodologies using different techniques to elute the lymphocytes from the SM (chemical or enzymatic), using different Mabs, inspecting the cells immediately or after storage and still others have analysed the lymphoid populations whilst in situ using histochemical techniques. Whilst most investigators have demonstrated a preponderance of $CD4^+$ cells in the SM, with ratios of $CD4^+ : CD8^+$ ranging from 4:1 to 14:1 [233], reports of normal proportions [234] or reduced numbers [215] have also been found. It has been proposed that these differences may also be due to sampling variations, since immunoelectron microscopic studies have shown that $CD4^+$ cells predominated in lymphocyte rich areas (where macrophage-like cells also reside), whilst $CD8^+$ cells predominated in transitional areas. These $CD8^+$ cells had the appearance of blast cells and were reported to be in close contact with macrophage-like cells [235].

A reduction in $T_{S/I}$ cells has also been reported in RA SM [236], which might in turn explain the diminished levels of $CD8^+$ cells in this tissue, since the $T_{S/I}$ are also said to be the main producers of IL-2, needed for $CD8^+$ cell function.

The interleukins IL-1 and IL-2 may also be important in determining the percentages of cells expressing the $CD4^+$ and $CD8^+$ phenotypes. IL-1 is reported to activate mainly $CD4^+$ cells, which in

turn generate IL-2, causing stimulation of CD8⁺ cells [237]. Whilst production of the former is reported to be elevated in RA [238], that of the latter is found to be reduced [239-241]. Furthermore, T-cells from the SM were found not to respond to exogenous IL-2 [240,241], probably due to prior activation. An average of 40% of T cells in the SM are activated [16] and whilst some authors report CD4⁺ to be the main HLA DR⁺ cells [242], others found CD8⁺ to be the main activated cells [209]. This could be to sampling differences and/or the fact that T cells can express both phenotypes following activation [243].

Whatever the explanation for the differences in the CD4⁺ and CD8⁺ cell representation in the SM, it has clear implications for the immunological activity in the RA joint, e.g. in synovial samples where T_{S/C} were present in similar proportions to those of T_H cells, follicular arrangements were rare and the different cell types were distributed diffusely throughout the synovium. Only in samples where T_{S/C} numbers were low, was there a gathering of cells into follicles [244]. The decrease in CD8⁺ cells in the SM is strange in view of the increase in the SF; one explanation is that there is local destruction of such cells as they pass through the SM by e.g. NK cells or humoral factors, e.g. IC or anti-lymphocyte antibodies.

Phenotypical analysis forms only an initial assessment of the regulatory pathways and of more importance is the study of whether or not the cells function in their phenotypic role.

b) Functional characterisation of T cells

In view of the B-cell hyperactivity in RA and the autoimmune nature of the disease, much emphasis has been placed on the measurement of suppressor T-cell function, since it is generally believed that a deficit is likely to exist here, rather than due to excessive helper T-cell function, since T_S activity is known to be important in the down-regulation of the immune response and maintenance of the "dynamic state of tolerance".

i) Suppressor cell function in RA

Evidence for defective suppressor activity in RA in various compartments is given below:

1) Direct evidence for defective T_S function in blood

Defective CON-A induced suppressor activity has been observed for suppression of B-cell synthetic responses [258], B-cell proliferative responses [245] and T-cell proliferative responses (in both MLC and mitogen-stimulated autologous proliferative cultures; [245-249]).

Of the few reports investigating Ag-specific suppressor cell activity, defective suppressor activity was found using ovalbumin-primed [250] and EBV-immune patients [251].

Defective (spontaneous) short lived suppressor activity has also been found in the blood [252].

2) Direct evidence for defective T_S function in SF and SM

Absent CON-A induced suppressor activity for suppression of proliferative MLC responses has been observed in RA synovial tissue [252].

Defective naturally occurring spontaneous suppressor cell activity for suppression of B-cell responses has been found in synovial tissue [279] and SF lymphocytes [231].

Deeming the lack of CON-A induced and spontaneous suppressor cell activity to be due to inadequate helper activity, the latter author [231] cocultured synovial fluid lymphocytes with peripheral blood T-cells (which incidently behaved as normal cells) only to find that Ig synthesis was enhanced. This was surprising, since most of the synovial fluid lymphocytes were of the CD8⁺ phenotype, suggesting a discrepancy between phenotype and function. This enhancement was referred to as "helper augmentation" and it was suggested that these CD8⁺ cells may be analogous to the murine contra-suppressor cells described by Gershon [253]. Another author obtained similar data using a purified CD8⁺ Leu 3a and B-cell population [215].

Defective Ag-specific suppressor activity has been observed in synovial tissue lymphocytes [254].

The lack of suppressor activity here leads to interactions between already activated T_H, macrophages and B cells leading to excessive IL-1 production (which in turn stimulates collagenase

release increasing degradative processes in the joint) and stimulation of IgM production which from a single knee can be 5-95 mg/day [113].

However, in studies where isolated $CD8^+$ cells have been examined, these cells have been reported to be very efficient suppressors [215,255], e.g. SF $CD8^+$ cells could suppress PWM-induced Ig synthesis as well as their peripheral blood $CD8^+$ cells. However, the same $CD8^+$ population was less able to suppress PPD-stimulated cultures (the same was found for blood $CD8^+$ cells), perhaps because PWM is a more efficient generator of suppressor activity [256].

Suppressor cell defects could result from a multitude of mechanisms such as the following: 1) a virus could preferentially modulate suppressor T-cells causing auto-Ag expression resulting in altered self-Ag, leading to auto-Ab formation that results in further inactivation of T_S cells and the expression of auto-reactive B-cell clones; 2) ICs could modulate $Fc\gamma$ receptors on T_S cells and thus alter their activity; 3) certain pathogenic auto-anti-id Abs could destroy T_S cells, particularly in view of the \uparrow B $CD5^+$ in RA, known to secrete auto-Abs; 4) altered T cell Ag's (e.g. defective glycosylation) could render T_S surface molecules antigenic and cause their selective killing; 5) lack of $T_{S/I}$ cells; 6) lack of $T_{S/I}$ factors (T_{SGF} , T_{SDF} , IL-2, γ -IFN) or factors from monocytes (PGE_2 and IL-1); 7) defective production of Ag-specific suppressor factors (T_{SF}) and/or Ag-non-specific suppressor factors (SIRS); 8) defective responsiveness in target cells (B, T and monocytes); 9) defective Ag presentation in

Ag-specific paths; 10) artifactual dilution of T_S by non- T_S lymphocytes; 11) increased $CD8^+$ CSC, or $CD4^+$, or $CD8^+$ T_C numbers and/or function; 12) anti- $CD8$ lymphocytotoxic Ab; 13) anti- $T_{S/I}$ Ab; 14) abnormal sensitivity of $CD8^+$ to inhibitory effects, e.g. PGE_2 ; 15) deletion of T-Fc γ cells via ANA of IgG class penetrating the cells through its Fc receptor and binding to their nuclei and abrogating spontaneous and CON-A induced suppressor cell function, as can occur in MCTD; 16) defective response to mitogens in "induced" suppressor assays; 17) by dilution of T_S regulatory factors either by metabolic breakdown or increased functional activity of B cells.

Evidence that some of these mechanisms are in operation in RA are given below:

a) Evidence for lymphocytotoxic Ab

Evidence for anti- $CD8$ Ab in blood in early RA (less than 3 months) has been noted [257,258] and in one paper, in chronic RA [259], but generally no anti- $CD8$ Ab are found in chronic or inactive RA [258]. Levels of anti- $CD8$ Ab are particularly high in RA with "malignant vasculitis" [260].

Evidence for anti- $T_{S/I}$ has been found in juvenile RA [261].

b) Evidence for defective responsiveness
of B cells to suppressive signals

Using a CON-A induced suppressor assay, such a defect was

observed in early and chronic active RA [245].

c) Evidence for abnormal sensitivity of CD4⁺ T-cells leading to defective T_S function

Certain glass adherent cells (of monocyte, T- or B-cell lineage) suppress mitogen- or EBV-stimulated T-cell mitogenesis by producing PGE₂. The latter inhibits γ -IFN production from CD4⁺ cells which is necessary for T_S function [262a,262b]. The CD4⁺ cells appear to be abnormally sensitive to the inhibitory effects of PGE₂ [262b].

d) Auto-anti-idiotypic Ab

Evidence has been found for RF idiotypic Ag on peripheral B- and T-cells in RA [263].

e) Defective responsiveness to CON-A

T-cells from RA patients blood have frequently been found to be unresponsive to CON-A, particularly SF lymphocytes [264]. This may be one reason why CON-A induced suppressor activity is frequently found to be defective in RA, particularly in SF [265].

Some papers suggest that the CON-A suppressor cell defects are more apparent in seropositive RA [266] or in early active disease [258,245], or in juvenile RA [267-269], or in the presence of RA with EAD, in particular "malignant arthritis" (RA with true vasculitis, [260].

The demonstration of a more pronounced suppressor defect in early active disease could mean that immunological dysfunction plays a role in the initial pathogenesis of RA. It could be that in RA SM, where T-lymphocyte levels are high, the lymphocytes may contain virus-like particles which could modulate auto-Ag of T_S cells leading to auto-Ab production with further inactivation of T_S cells with subsequent expansion of auto-reactive B-clones, with inflammation being maintained by the auto-antigenic changes caused by the virus and perpetuated by ROS.

There is some evidence that certain T_S cells inhibit ROS production, thus theoretically a defect in these T_S cells could raise ROS levels which could then inhibit other T_S cells and worsen the immunodysfunction and perpetuate inflammation [270].

However, not all the literature agrees that defective suppressor activity occurs in RA.

Other studies have shown no significant CON-A induced suppressor cell defect for suppression of B-cell responses [258,260] or T-cell responses (mitogen-stimulated proliferation, MLR, mitogen-stimulated AMLR) [245,247,248,271,272], probably due to heterogeneous disease activity and not taking into consideration drug therapy, since these studies used patients who were taking steroid or sulphydrate drugs.

Other studies have shown excess short lived suppressor activity in peripheral blood, but again bad choice of patients (mild or

inactive disease and the use of sulphydrate drugs and/or low dose steroids) may account for this [271,272].

Still others have shown excess radiosensitive PGE_2 stimulated CD8^+ T-cell and non-T-cell spontaneous suppressor activity for regulation of IL-2 production in RA [273]. In this study none of the patients had taken second line drugs for at least three months, and NSAID treatment was withdrawn 24 hours before the analysis. It is not known whether there is excess number or potency of such cells, or whether these cells are abnormally sensitive to the stimulatory effects of PGE_2 [273]. It has been suggested that this excessive suppressor function could account for reduced IL-2 production in RA [273], and could partly explain the in vitro hyporesponsiveness of RA T- and B-cells to mitogens and Ag's [262d].

Excessive CON-A induced suppressor activity of MLC [274] and excessive sensitivity of B-cells to HAGG-stimulated T_S influences in RA blood has been observed [272]; however in the former paper the patients were on sulphydrate drugs, and in the latter on a variety of medications.

In three studies a variety of functional suppressor assays were assessed in the same individuals: 1) excess CON-A induced mitomycin-C sensitive, but reduced mitomycin-C resistant suppressor activity has been found for inhibition of B-cell responses in an allogeneic system

[269]; 2) defective CON-A induced suppression of B and T cell responses was found in early active RA, but in chronic active RA, despite normal CON-A induced suppression of T cell responses,

defective suppression of B cell responses was observed [245]; 3) excess short lived suppressor activity, normal or defective CON-A induced suppression of T-cell responses, and excessive sensitivity of B-cell to HAGG-stimulated T_S cell was observed [272].

The finding of a fluctuation in CON-A induced suppressor activity with disease activity may suggest that such defects reflect secondary manifestations of the disease rather than significant pathogenetic events. The finding of excess suppressor activity for regulation of certain functions and defective suppressor activity for regulation of other functions is not contradictory, since it can be hypothesised that such cells regulate different circuits independently of each other.

Since rheumatoid inflammation starts from the SM, the study of SM or SF lymphocytes may be more relevant than blood lymphocytes - however most studies use peripheral blood lymphocytes because of the difficulties in obtaining the former two types of lymphocytes, and even greater difficulties in obtaining suitable control lymphocytes; thus on the assumption that peripheral blood lymphocytes function similarly to joint lymphocytes, the former are frequently utilised.

ii) Helper cell function in RA

There is now evidence for a complex battery of T_H cells for up-regulation of the immune response including antigen specific T_H cells providing help by recognition of carrier determinants on Ag (as opposed to the haptenic determinants recognised by B cells), providing

help by recognition of Ig determinants (isotype, allotype and idiotype specific T_H cells) and antigen non specific T_H cells induced by mitogens. All function directly or indirectly via antigen specific and/or antigen non specific T_H soluble factors.

Experiments analysing T_H function in RA have likewise produced conflicting data.

In peripheral blood, T_H function has been reported to be defective [215,275], normal [231], or increased [276]. One report examined the spontaneous generation of IgM RF production in vitro and reported that T-cells from RA patients produced enhanced help for auto-Ab production which was not HLA restricted, thus normal as well as RA B-cells could be induced to generate RF when co-cultured with RA T-cells [277].

In the joint, SF T_H cells have been shown to have a low ability to cooperate with PWM-induced RF secretion [278].

Synovial tissue lymphocytes are said not to differ from normal peripheral blood lymphocytes in helper activity for Ig production [279], whilst others found it to be impaired compared to autologous peripheral blood T-cells [231].

In studies examining isolated $CD4^+$ cells, contradictory data has been obtained, Leu 3a⁺ cells have been found to be weak augmentators of the Ab response [215], whereas SF $CD4^+$ cells were found to be better augmentators of PWM-induced help than the corresponding unfractionated T-cells, perhaps due to suppression by PWM-stimulated

CD8⁺ cells of CD4⁺ function in the unfractionated preparation [255,256].

Functional assays have clearly produced much conflicting data. This is probably because investigators have tried to analyse complex regulatory systems with relatively simple fractionation or culture systems, together with the fact that more and more evidence is accumulating regarding the heterogeneity of the two main T-cell subsets (CD4⁺ and CD8⁺ cells), e.g. a much greater degree of heterogeneity than was previously appreciated exists in the CD8⁺ subset in which the existence of suppressor precursor T-cells, suppressor activator and effector T-cells have been postulated [280], together with antigen specific T_S cells (providing suppression by recognition of carrier determinants on Ag), antigen non specific T_S cells, T_S providing suppression by recognition of Ig determinants (isotype, allotype and idiotypic specific T_S cells). All function directly or indirectly via antigen specific and/or non specific T_S factors.

Also, T_{S/I} activity is said not to exist in unprimed T-cells [281], whilst others believe it does [198,210]. These differences may be due to the former group using an Ag to examine such an interaction, whilst the latter used PWM.

Until T-cell populations are more clearly defined phenotypically and functionally, and the various interrelationships better established, it is probable that contradictory data will continue to emerge from various studies.

8) Cytokine production

Regulation of the immune response is achieved in part by the release of soluble mediators called cytokines produced by mononuclear cells. Recent evidence has indicated imbalances in the production of these cytokines in RA which may be relevant to the immunopathogenesis of the disease. The cytokines include antigen non specific factors, e.g. IL-1, IL-2, γ -IFN and antigen specific factors, e.g. T_HF and T_SF .

IL-1 production

In response to Ag or mitogens, monocytes, macrophages and dendritic cells produce IL-1 [40,282] a polypeptide of MW 15 kd. This then induces T-cells of both $CD4^+$ and $CD8^+$ phenotype to produce IL-2, although $CD4^+ T_{S/I}$ are thought to be the main producer [236]. IL-2 synthesis is necessary for lymphocyte proliferation [283] and stimulates T-cell γ -IFN production [284], another cytokine which regulates the immune response.

IL-1 production has been demonstrated in the RA SM [40,285] and SF [286]. IL-1 has several activities relevant to the pathological changes seen in the rheumatoid joints, e.g. causing collagenase and PG secretion by synoviocytes [40], the proliferation of fibroblasts [287], the induction of chondrocytes to degrade the cartilage matrix [288] and stimulates bone resorption and T_H and B cell activity. IL-1 production is raised from peripheral blood monocytes [289-291] and this may account for the systemic features of RA, namely neutrophilia, fever, and the acute phase response [292].

IL-2 production

IL-2 is a polypeptide of MW 15 kd produced by a variety of cell types including $T_{H/I}$, $T_{S/I}$, NK cells and $CD8^+$ T-cells.

In vitro IL-2 production by rheumatoid peripheral blood lymphocytes has generally been found to be deficient [239-241,273,289,293,294], however normal [295,296] or higher than normal levels [273] have occasionally been reported. These discrepancies may be partly due to methodological differences, but could also reflect the broad clinical and pathological spectrum of RA, the practical difficulties associated with the use of ARA criteria in the clinical setting to diagnose RA and finally the difficulty in defining severity of the disease. Raised IL-2 production in RA could be an in vitro artifact when irradiated MNC are used since this process inhibits $CD8^+$ and non-T cells that regulate IL-2 production [273]. Defective IL-2 production could be the result of poor IL-1 production from monocytes (unlikely in RA since this is high), reduced IL-2 transcription due to an invading virus, inability of T cells to respond to mitogen or IL-1, inhibition of IL-2 receptor expression or binding or signal transduction needed for up-regulation of IL-2 production, incapacity to make or secrete IL-2, or increased sensitivity of $CD8^+$ T-suppressor cells that regulate IL-2 production to stimulatory signals - e.g. PGE_2 [262d] - particularly in view of the already reduced $T_{S/I}$ numbers (the main producers of IL-2), and in some people it could be due to excess inhibition of IL-2 producing cells by more numerous and/or more potent spontaneous radiosensitive,

PGE₂ induced, non-T and CD8⁺ suppressor cells [273].

Studies investigating whether there is a relationship between IL-2 production and disease activity have produced conflicting results. One study showed IL-2 production correlated inversely with disease activity [273], whereas another suggested IL-2 levels correlated positively with disease activity [296]. However these studies were performed with small patient groups which were clinically and therapeutically diverse. In a third report using large patient groups which were clinically and therapeutically homogeneous, IL-2 production was increasingly defective from inactive, to active, to active with EAD disease [294]. Defective IL-2 production could account for the in vitro hyporeactivity of T-cells, since IL-2 corrects depressed AMLR reactions [297] and could account for the defective T_S activity in the rheumatoid joints, where deficient IL-2 production from synovial lymphocytes has been observed [241].

Most reports view autoimmune disease as being related to defective IL-2 production; however, equally, a recent paper suggested that one could interpret the following findings: 1) reduced activity of a 60-70 kd IL-2 inhibitor in sera of 50% of RA patients, 2) the ability of cyclosporin A and steroids to stimulate IL-2 production in vivo but inhibit it in vitro and 3) defective IL-2 production - as in fact reflecting IL-2 hypersecretion, such that defective IL-2 production in vitro could simply imply: 1) in vivo activation preventing the cells from responding further, 2) elevated binding and internalisation by CD25⁺ cells and 3) excess T_S activity [298].

γ -IFN production

IFNs have anti-viral properties and can inhibit cell division and regulate immune responses by stimulating NK activity and inhibiting other cell mediated immune responses. They also stimulate phagocytosis and cause up-regulation of HLA-DR expression.

γ -IFN (type II) is a polypeptide of molecular weight 20-25 kd produced by activated $T_{H/I}$, $T_{S/I}$ and NK cells.

Defective γ -IFN production has been observed from RA peripheral blood and SF T-lymphocytes [241,299]. This could be due to the defective IL-2 production, but is unlikely to be due to the inhibitory effects of monocyte generated PG's since removal of adherent cells does not improve its production [241]. Defective γ -IFN production could also result in B-cell hyperactivity with the resultant immunological injury seen in the rheumatoid joints.

On the other hand, γ -IFN has been noted in RA SF, and stimulated SF T-cells were said to produce normal amounts of γ -IFN [299b].

Ag specific T_S and T_H factors

Such factors have been described in man, but definitive molecular characterization must occur before these factors can be assigned a role in immunoregulation and only then will it possible to look for excess T_H and reduced T_S factors that could theoretically occur in RA.

Thus it seems that wherever you look for an immunoregulatory defect in RA, you find one - which is perhaps not surprising in view of the complex interrelationships between all these regulatory pathways.

IN VITRO FUNCTIONAL MODELS FOR SUPPRESSOR CELL ACTIVITY

With the introduction of antigen to the immune system, effector mechanisms and positive (helper) and negative (suppressor) feedback control mechanisms are activated to precisely regulate the immune response, and together decide the nature and intensity of the response. The magnitude of the response depends on several factors, e.g. degree and mode of antigen stimulation and inherent proportion of immunocompetent lymphocytes. This is not a unique occurrence since all complex biological processes are controlled by impressively precise mechanisms, e.g. hormonal synthesis, C¹ activation, etc., but it can be seen that it is particularly important when dealing with systems such as the immune system with its capacity for producing potent inflammation and because it must face a multitude of stimulations throughout the organism's life.

Nature's control mechanisms utilize her "tug-of-war" approach, with opposing forces often simultaneously operating, producing a net immunological effect, such that for "every immunological effector action there is an almost equal but opposite regulatory response".

Such a system offers a mechanism for very sensitive regulation. Thus it appears that any immune response generates an antiresponse and thus any immunocompetent cell is controlled by other cells with antagonistic action and each may have its own specific route of suppression, the ultimate aim of the immune system being to allow efficient response against foreign determinants but to prevent any inappropriate responses to self antigens. Many different and complex immunoregulatory systems have been described for the human immune response with numerous suppressor influences in existence, each directed at its own specific target cell. Thus immunosuppression can be regarded as a physiological homeostatic mechanism to maintain the immune system with an immune response representing a breakdown in homeostasis. The readily proposed theoretical mechanisms by which defects in immunoregulatory cells might produce disease have now been established as important in numerous clinical studies; thus immunoregulatory action can be inappropriately excessive or restrained and effector cells may be both unusually sensitive or insensitive to control mechanisms. Thus autoimmune diseases have been found to be associated with decreased suppressor cell activity and B-cell hyperactivity, and conversely excessive suppressor cell activity and B- and T- cell hypoactivity is found in diseases associated with immunodeficiency, such as common variable immunodeficiency disease, disseminated fungal infections, Hodgkins disease and sarcoidosis.

The concept of immunoregulatory systems directly dependent on cells has emerged in the last twenty years. This was based on early experiments by Gershon [300-302], the founder of suppressology, in which mice were made unresponsive by injection of high doses of sheep red blood cells and their T cells were then capable of suppressing Ab synthesis when transferred to normal recipients. It was the existence of inbred strains of mice that permitted the use of these adoptive transfer experiments and simplified experimental protocols designed to investigate the role of suppressor and helper cells in immunoregulation [303,304]. With such techniques suppressor cells have been found to be important in the development and maintenance of immunological tolerance [305], allotype suppression [306], antigenic competition [302] and in some cases H-2 linked Ag-specific unresponsiveness [307], graft versus host disease [308] and delayed hypersensitivity [309]. The current view is that suppressor cell regulation of the immune response involves an intricate network of different systems rather than one single system [310].

Essentially every major cell type that is responsible for an immune response may also be capable of exerting regulatory functions. There is good evidence that under certain conditions B cells [311], null cells and natural suppressor cells [312] and adherent cells of both the polymorphonuclear [313] and more especially the monocyte/macrophage series [314,315] can show an immunosuppressive function for different immune reactions, but the major suppressor cell

type is the T cell. Apparently distinctive T_S cells have been identified in man, although there is probably some overlap identified in single function studies. T suppressor cells that have been identified include 1) spontaneously active T_S cells [316,316b], 2) short-lived T_S cells [317-319], 3) spontaneously induced T_S cells [320], 4) prostaglandin producing T_S cells [61,321], 5) antigen specific HLA restricted T_S cells [322], 6) T_S cells that recognize Ig determinants, e.g. isotype specific T_S , allotype specific and idiotype specific T_S cells [323], 7) alloantigen specific [324] and 8) contrasuppressor T_S cells [323] within the OKT4 and OKT8 population, 9) memory T_S cells and 10) antigen non specific inducible T_S cells [317].

Analysis of immunoregulatory mechanisms in man has been difficult to accomplish since many of the experimental approaches used in mice cannot be employed in man. Numerous in vitro models have now been designed to investigate suppressor cell activity in man with variations in methodology but all based on a few basic principles. Given the large number of assays now available it would be impossible to describe each of them. Thus representative systems will be described in detail to illustrate the principle on which they are based.

Model A

"Based on adding putative spontaneous suppressor cells in fractionated or unfractionated mononuclear cell preparations to a system capable of a positive immune response, and examining whether the final response is affected by the presence of the cells under test" [316,316b].

This approach is usually used where excessive suppressor activity is expected. The most frequently used indicator systems are B cell systems such as inhibition of mitogen or antigen stimulated allogeneic B cell blastogenesis, IgG synthesis or secretion; or mitogen or antigen stimulated autologous B cell responses. Spontaneous suppressor cells have been identified in T and non-T populations and adherent cells and seem radio-sensitive, steroid resistant and Fc γ receptor positive.

An example of this model is the finding that PBMNC or isolated T cells from some patients with common variable hypogammaglobulinaemia inhibited normal B cell maturation and Ig synthesis to a greater extent than cells from healthy controls [325].

The disadvantage of this model is that results are very variable from individual to individual, so its use in clinical situations is difficult, although it is present in some perfectly healthy humans [316b,325b,325c] and may reflect both antigen specific and non-antigen specific suppressor cells.

Model B

"Based on removing a particular cell from a system capable of positive immune response and observing whether this results in an enhancement of the immune response". Such cells appear to be spontaneous, but unlike model A this approach can be used in clinical situations where either excess or deficient suppressor activity is likely.

An example of this model is the short-lived suppressor cell assay described by Bresnihan in man [317-319]. In this assay short-lived suppressor cells are eliminated or inactivated in a preliminary culture step (usually 24 hours) and the effect of this manipulation on subsequent proliferation in response to mitogens is investigated; usually submitogenic doses are used since strong mitogenic stimulation results in loss of this suppressor effect. The precise nature of such cells is not known and in fact no suppressor cell subpopulation has been isolated and demonstrated to be short-lived [326]. However there is ample evidence for the existence of such cells, e.g. in murine systems suppressor cells are short-lived in culture [327] and in studies of fungal infections in man there are abnormally high numbers of suppressor cells that appear to be short-lived in culture [328]. The activity falls with age [329]. Cell viability studies have shown no significant differences in cell survival in cultures with or without preincubation, but presumably the number of short-lived

suppressor cells is only a tiny proportion of total MNC, otherwise no immune response would ever be possible. The phenomenon does not seem to be due to soluble suppressor factors or aggregation of cells, but an actual suppressor cell effect, since suppression is still observed after resuspension of cells in fresh medium after the preincubation step [319]. Short lived suppressor activity is thought to give an indirect estimate of spontaneous suppressor activity.

This activity does not appear to be an artifact and is a simple and convenient assay to use and is therefore widely used in autoimmune conditions where defective suppressor activity is expected and immune deficiency conditions where excessive suppressor activity is expected. Various workers have ruled out the possibility that reduced suppressor indices observed in autoimmune disease, using this assay, are an artifact resulting from reduced mitogen responses and have shown that it is a genuine effect not attributable to low mitogen responses per se. A disappointment with this assay is that it is not antigen-specific and certainly does not reflect the complete regulatory potential of the PBMNC under study.

Model C

a) "Based on adding mitogen-induced antigen nonspecific MHC unrestricted putative suppressor cells to a second indicator culture system capable of a positive primary (e.g. PFC to sheep red blood cells), or secondary immune response and comparing the outcome to the

effect of adding cells not exposed to the mitogen in a second culture".

This double culture method is usually adopted in cases where defective suppressor activity is expected. Of the various mitogens that can be used for induction of suppressor activity, CON-A appears to be the best [317]; others include PHA [330] and PWM [331]. The different mitogens appear to activate different populations of T_S cells to suppress in similar indicator systems, e.g. PWM stimulated $CD8^+$ cells that inhibit T cell proliferation are $CD28^- CD11^+$ [200], but those stimulated by CON-A to inhibit proliferation are $CD11^- CD28^+$ [332].

Unfortunately this method measures only antigen nonspecific activity, which may not be biologically relevant, and it is unknown whether inducible suppressor activity is related to the spontaneous suppressor capability of MNC which is clearly more relevant to disease processes since measurement of the latter is more indicative of the in vivo situation.

An example of this model is the CON-A induced suppressor assay. As well as its ability to stimulate T_H and T_C function and to induce T cell proliferation, CON-A can stimulate a variety of suppressor cell precursors to become active and different functional types of suppressor cells. The different functions reflect the enormous heterogeneity within the induced suppressor population. Depending on

the indicator systems used one can monitor the suppressor activity of particular subsets of CON-A induced suppressor cells since all possible types are produced at once and there is some evidence that even in one assay different phenotypic cells can act together. Such induced suppressor cells can be detected by inhibition of T or B cell blastogenesis or inhibition of B cell synthetic processes; although the degree of suppression is similar in most assays (60-100%), more variability occurs using allogeneic cells as a stimulator because such a response depends on HLA-D incompatibility which is variable between each individual. Assays not involving allogeneic cells are therefore preferable because they are easier to perform and interpret.

It is now agreed that the major lymphoid subsets activated by CON-A are a heterogeneous population of T_S cells including those that 1) are HLA-D restricted in their MLR suppression; 2) inhibit MLR; 3) block all MLR proliferation; 4) block T cell responses to antigens (e.g. PPD and Candida Albicans); 5) block T cell responses to mitogens; 6) inhibit mitogen induced cytotoxicity; 7) block cytotoxicity induced in MLR; 8) inhibit macrophage inhibition factor action; 9) inhibit T cell responses to altered self; 10) block B cell proliferative responses to antigens; 11) block B cell proliferative responses to antigens; 12) block B cell synthetic responses to antigens; 13) block B cell proliferative responses to mitogens; 14) block B-cell synthetic responses to mitogens, reviewed by Dwyer [333]. Wherever non-T cells have been said to be induced by CON-A, this may

merely be because all other suppressor cells in non-T populations coexist with the putative precursor suppressor cells that can be activated by CON-A.

CON-A stimulates all available suppressor cells (reflecting latent immunological control), so results from this assay generally show less scattering and are more easily applicable to clinical situations than the previous models. In normal individuals the response of fresh cells co-cultured with CON-A treated cells is consistently decreased compared to responses with untreated cells and this inhibition is interpreted as CON-A generated suppressor activity (see discussion for more details).

b) "Based on adding alloantigen induced putative suppressor cells to a second indicator culture system capable of a primary or secondary immune response, such cells are produced in allogeneic mixed lymphocyte reactions in MLC" [324,334].

Since such suppression is HLA-D restricted, it can be difficult to interpret and a lack of suppressor activity may simply be due to insufficient differences in HLA antigens between the two donors. The activity is alloantigen specific, is insensitive to radiation and mitomycin C treatment and could be an in vitro artifact since IL-2 inhibits its action. Such cells are induced by a T cell differentiation factor which is antigen nonspecific and MHC unrestricted and functions via transiently expressed receptors. MLR T_S cells function via T

suppressor factor production. It has been suggested that CON-A or alloantigens could induce suppressor activity by activating latent intracellular viruses with concomitant immunosuppressive effects. If so, such a virus(es) must be widely distributed in the blood of healthy subjects [317,317b].

c) "Based on adding antigen-induced putative suppressor cells to a second culture system capable of an immune response, e.g Ig synthesis or lymphocyte proliferation". Antigens that have been used include purified protein derivative of tuberculin (PPD) [335], staphylococcal enterotoxin (SEB) [336], mycobacterial antigen [337], tetanus toxoid (TT) and ovalbumin.

The disadvantages of this antigen specific system compared to the CON-A assay is that responses are more variable and a longer culture period is required. The advantage is that it is of course antigen specific and physiologically more relevant. Use of this assay with systems offering a possibility of studying a primary immune response allow the study of early stages of B cell activation in which T cell regulation is particularly essential. Once activated and expressing factors of maturation and differentiation, B cells may be less subject to antigen specific T_S control, but more under the regulation of their soluble factors.

d) "Based on assaying for antigen specific T_S cells present in immunised or primed subjects" [250,251,338]. Systems offering the possibility of studying a secondary immune response (using primed

cells) are more complicated to interpret due to the production of the more complex array of regulatory T cell subsets and possibly even CSC cells too.

Model D

a) "Based on assaying for suppressor cells that evolve after variable periods of time in FCS stimulated culture in the absence of mitogen. These are called culture induced or spontaneously induced T_S cells".

An example of this model is that PMNC preincubated for two days [339] or seven days [320] caused inhibition of Ig synthesis from autologous lymphocytes stimulated with PWM, but not allogeneic cells. The culture induced suppressor cells appear to be a proliferating T cell population induced by a spontaneous autologous mononuclear lymphocyte reaction (AMLR) necessary for maintaining self tolerance.

The disadvantage of this assay is that it is not very reproducible individual to individual, so it is not very applicable to clinical situations.

b) "Based on assaying for suppressor cells produced in a true AMLR where proliferating T lymphocytes are added to a radiated autologous non-T lymphocytes, by assaying their effects on proliferative or synthetic responses of autologous or allogeneic cells".

Interestingly CON-A induced suppressor cells for suppression of PFC [340a] and T cell proliferation [340b] appear to be drawn from the same population of T cells activated in the AMLR. The AMLR produced T_S cells appear to be $CD8^+ DR^-$ and require $HLA-DR^+$ non-T cells for their generation and show HLA-DR restricted activation but not action, and they are mitomycin C and radiation sensitive. The target cell is a B lymphocyte and such T_S cells suppress by cell contact in a cytostatic mechanism and not by soluble factors. The CD8 antigen is not involved in their action and they can inhibit antigen or mitogen stimulated proliferation or PWM stimulated Ig synthesis. Since IL-2 stimulates their action, this activity appears not to be an in vitro artifact due to IL-2 consumption.

Finally suppressor cells can be induced by culturing PBMNC with suppressor T clones or with r-IL-2 [341] or following drug treatment, e.g. spirogermanium [342], or even by mitogen not requiring a preincubation period [343], but the latter is difficult to interpret. Suppressor function can also sometimes be shown in PWM-stimulated Ig cultures at high T/B ratios [344].

After any of these assays it is usual to try to identify the cell population(s) or subpopulation(s) being monitored. Detecting the suppressor cell population in mitogen stimulated assays has proved difficult since heterogenous populations probably act simultaneously in any one indicator system, which probably accounts for the

discrepancies noted in the literature using even the same indicator system. One can try to identify whether the suppressor cell is adherent (to plastic, glass, sepharose G10, nylon wool) or nonadherent and whether it is autorosetting or not; if adherent to glass does it mediate its effect via prostaglandin? One can see whether it is radiosensitive, steroid sensitive or prostaglandin sensitive. One can also see whether it is a T or non-T cell and if a T cell what phenotype it has. One can attempt to decipher what cell and soluble factor requirements the particular suppressor cell being monitored has and what target cell it affects and its mode of action, i.e. whether via direct cell-cell contact and/or soluble factors and whether it is a cytotoxic or cytostatic mechanism.

All the various T_S cells described are suggested to be induced via unique suppressor cell cascades and T_S circuits involving T_S inducer, transducer and effector cells and Ag and non-Ag specific soluble suppressor factors.

Despite a plethora of papers on the subject, there is still a great lack of understanding of suppressor cells, and the relationship between the various T_S circuits is not known. It can be seen that most in vitro models detect antigen non specific HLA unrestricted suppressor activity, but in vivo suppression is probably mediated via Ag specific MHC restricted suppressor cells, so it remains to be seen if such in vitro models are relevant, however on the assumption that they may be relevant and because few Ag specific assays are available,

they continue to be used to provide information about normal and abnormal human physiology.

AETIOLOGY OF RA

That a chronic immune reaction is occurring in RA is clear, but despite many years of intense research, the aetiology of RA remains obscure. It is probably multifactorial and despite suggestions that it may result from a generalised disturbed microcirculation [345] or even to food intolerance [346], the generally accepted view is that RA arises out of a deranged regulation of the immune response to an antigenic stimulus in a genetically susceptible host. This could take either of the following two courses:

- a) inability of an individual to eradicate the offending antigen such that it persists, resulting in chronic immunity or,
- b) a failure to regulate the immune response after a normally transient stimulation by antigen, taking the form of either i) an inadequate response leading to a state of "immune deficiency", or ii) inappropriate intensity leading to a "hyper-immune response" [120].

Possible identifications for the offending antigen include:

- a) microbial infection, e.g. streptococci, diphtheria bacilli, *S. aureus* or a sporochaete from a tick, or mycoplasma.
- b) Viral infection, e.g. EBV or other slow acting viruses. Cytoplasmic inclusions in the SM resembling the nucleoplasmid of paramyovirus have

been seen in RA, but there is no evidence that these inclusions were in fact viral.

c) Aggregated IgG.

d) Components of synovial cartilage and collagenous tissues.

Looking at the possibilities in more detail, it must be said that a microbial infection remains an attractive proposition. However as yet no firm microbial evidence has been produced to sustain such a view. Circumstantial evidence favouring this idea is however available, e.g. chronic arthritis can accompany a number of natural and experimentally induced infections in animals, e.g. pannus formation along with other features of rheumatoid synovitis are seen in some streptococcal infections of rabbits [120]. Bacteria can also cause some forms of arthritis, e.g. infections by *S. aureus* [347]. Studies to isolate bacteria from rheumatoid synovial tissue have met with little success [347], although antibodies to *proteus mirabilis* have been found in some patients. Lyme disease, a systemic, inflammatory disorder also causes some changes in the synovium similar to those seen in RA [348], and is now thought to be transmitted by the tick *Ixodes damini* [349] which carries the presumed infective agent which is a spirochaeta (*Borrelia burgdorferi* [350]). In swine the *Erysipelothrix* organism produces arthritis which clearly resembles the human disease and classically, subacute bacterial endocarditis is accompanied by RF production, levels of which fall off once the

disease is resolved. It has been suggested that the anti-IgG activity may arise as result of cross reactivity with bacterial antigens [119], although more recently it has been suggested that the autoantibody is anti-idiotypic for virus induced anti-FC antibody [351].

More likely candidates are viruses, and particular interest has been expressed in a role for infection by Epstein-Barr virus (EBV). This is based on a high incidence (67%) of antibodies in RA patients directed against EBV-associated nuclear antigens (RANA's) compared to 8% in normal subjects [352,353], and even higher incidences have been detected by others [354], but the specificity of their assays has been challenged. RA lymphocytes can be stimulated by EBV (a polyclonal B cell activator) to produce IgM RF and in fact in greater amounts than control lymphocytes [352], the RA lymphocytes transform more spontaneously and show more rapid transformation with EBV than normal cells, which may suggest defective T suppressor function [355]. Since anti-RANA antibodies occur in other diseases too, the data is inconclusive regarding the importance of EBV in RA. The idea that RA is initiated by some sort of polyclonal B cell activator causing disturbed IgG production including autoantibodies is obviously quite an attractive one. However the rather restricted nature of the immunoglobulins produced in RA [111-113] would argue against such a suggestion.

Although some of the above reagents (e.g. bacterial wall peptidoglycans and mycoplasma containing complete Freud's adjuvant)

have been used successfully in generating models of RA in laboratory animals, attempts to isolate these organisms from synovial tissues in RA have proved unfruitful [356].

At present there is insufficient evidence to cast any of the above microorganisms in an aetiological role. However, given that such a wide variety of agents can produce arthritis, it is obvious that other factors must be involved in allowing the full expression of rheumatoid disease.

IMMUNOGENETICS

When it became known that mice with particular antigens were susceptible to certain diseases and that there were genetic links between histocompatibility antigens and immune responses, researchers began to study the frequency of HLA-antigens in human diseases, particularly those of unknown aetiology with an inflammatory or immunological component and with clinical or epidemiological evidence of a genetic component.

The major histocompatibility complex (MHC) region in man is found on the short arm of chromosome 6 and includes the HLA region, which is a cluster of genes with different properties, class I genes determining serologically defined antigens, while class II genes encode antigens important in the generation and regulation of immune responses and class III genes encode complement components, genes

throughout the cluster being associated with susceptibility or resistance to diseases. The MHC region thus plays a dominant role in determining the immunologic potential and performance of an individual and in several diseases an association has now been found between certain HLA antigens. The first demonstration of a relationship between genetics and the rheumatic diseases was when a strong association was found between HLA-B27 and ankylosing spondylitis [357].

The immune response genes encoded by HLA-DR antigens of the class II MHC region control among other things T-B cooperation, suppression of the immune response, and antigen-induced antibody production leading to the proposal that RA might result from an abnormal immune response in a genetically susceptible individual to a perfectly normal pathogen. However it has been shown that the increased incidence of HLA-DR4 in RA patients is only observed in seropositive disease and that patients with seronegative RA do not differ from normal healthy controls [358]. Thus possession of the DR4 haplotype is now thought to be related only to the degree of disease severity and not to an individual's susceptibility to RA [359]. This idea is supported by the observation that 95% of Felty's subjects are HLA-DR4 positive, this being the most severe type of RA found.

Possible mechanisms involved in HLA-disease associations include a) disease susceptibility genes - where the HLA antigens may play no part in the disease process but may act merely as markers for disease

susceptibility; b) molecular mimicry - where an individual may become infected with a microorganism whose surface antigens are similar to the host's HLA antigens resulting in non recognition of the organism as foreign, leading to a state of tolerance to low grade infection; alternatively an immune response may be mounted, but might cross-react with similar HLA agents on the host's tissues producing an autoimmune effect; c) altered self - in which HLA antigens could be altered by infectious agents, drugs or other environmental agents, resulting in autoimmune reactions; d) membrane receptors - where certain HLA antigens may act in competition with immunoregulation or inflammatory mediators or hormones, resulting in disease; they could also serve as receptors for a virus or an environmental toxin; e) they could simply reflect genes for producing mediators, hormones and amplifiers, e.g. complement components or enzymes or f) reflect markers for unknown genes. When assessing which hypothesis best fits a particular disease, it must be able to account for those individuals with the disease who lack the associated antigen.

If HLA associations are simply a reflection of disease susceptibility or immune response genes being measured by linkage disequilibrium, then the HLA antigens themselves and their precise definition, chemical characterization and tissue distribution will be of little consequence in the context of HLA associated studies. However, should they be directly involved in any of the disease processes, then precise antigen definition both serological and

chemical, together with tissue distribution will be vital for a full understanding of the disease mechanism. If we ever can by genetic markers tell who is going to develop RA, then of course the whole face of modern rheumatology would change, because preventive medicine would become the main research and clinical drive. It is encouraging that RA should have an association with genes of the DR region of HLA, since being concerned with the regulation of the immune response it supports the idea that RA is a disease of deranged immunoregulation.

CHEMOTHERAPY IN RA

RA occurs not as a single entity, but as a spectrum of disease activity ranging from inactive or mild to severely active erosive disease. This necessitates the use of individual treatment regimes for each patient depending on the stage and severity of the illness. The chemotherapy may modify the disease process or control specific manifestations of it, but it is not curative, although in some patients the disease may remit. The drugs used in RA may be divided into the following categories:

- 1) Analgesics.
- 2) First line drugs, which are the non-steroidal anti-inflammatory drugs (NSAID).
- 3) Corticosteroids.
- 4) Second line drugs, which are the anti-rheumatic drugs.

5) Third line drugs, which are the cytotoxic drugs.

Agents within each group may be used singly or, more usually, in combination with those from other groups.

1)Analgesic and 2)NSAID therapy

Initial therapy may begin simply with analgesics for pain relief, but may later necessitate NSAID treatment (aspirin and salicylates used since 1899 and newer drugs based on derivatives of phenylacetate, e.g. phenylbutazone, or derivatives of acetic acid, e.g. indomethacin or derivatives of propionic acid, e.g. ibuprofen, or derivatives of phenylalkanoic acid, e.g. naproxen) to achieve pain relief (by their analgesic action) and some reduction in the inflammation causing joint swelling (by their anti-inflammatory action) and reduction in fever (by their anti-pyretic action), thereby allowing better joint function and improving the patients well-being.

NSAID's do not cure or permanently reverse the disease process and so do not reverse the arthropathy, but, because they allow better joint function and are well tolerated and help the clinical situation, are widely used.

The ability of NSAID to act as ^{anti-}inflammatory agents is due to their ability to inhibit prostaglandin synthesis, which in vitro occurs by suppression of the cyclooxygenase pathway [360]. Raised PG levels, especially PGE₂, would otherwise synergise with other

inflammatory mediators, e.g. C5a and bradykinin, together with acute phase proteins and increase vascular permeability and enhance blood flow to produce the five cardinal signs of inflammation. The use of such agents in RA is questionable in the light of some data showing the importance of PG's in the suppression of the immune response (see section on immunoregulation), and the fact that inflammation is an important physiological event involved in the "healing" process, so NSAID treatment could possibly worsen the situation, and also because salicylates may even reduce glutathione levels important for attempting to maintain surface -SH levels. Although all NSAID drugs cause inhibition of cyclooxygenase in vitro, they vary greatly in their potency [361], and this variation does not correlate with their rather equivalent clinical efficacy, suggesting that their action may be more than simple inhibition of prostaglandin synthesis, e.g. aspirin at 600 mg/day cannot produce antiinflammatory effects in RA SM but totally abolishes cyclooxygenase activity, e.g. salicylate does not greatly affect PG synthesis, but is as good as aspirin in vivo. Because PG's are known to have striking immunoregulatory effects, e.g. stimulate spontaneous suppressor activity [362a], inhibit CON-A induced suppressor activity [264,362b], the fact that NSAID do not have a profound effect on the RA process (despite inhibiting PG-dependent spontaneous suppressor activity [362a] and stimulating CON-A induced suppressor activity in vivo [362b] and in vitro [362a,362c], and inhibiting IgM RF synthesis in vitro [262c]) suggests that it is unlikely that PG modulation of the immune response

plays a major role in the pathogenesis of the disease.

The other mechanisms of action of NSAID are thought to center on PMN function. Both lysosomal enzyme release [363] and oxidative metabolism [364] have thus been shown to be suppressed in vitro by NSAIDs. Conversely, aspirin has itself been found to induce gastric mucosal injury, thought to be due to its stimulation of free radical and lipid peroxide generation, so it is conceivable that this may occur in RA joints too, worsening the situation.

The mechanism of action of these drugs in inhibiting aspects of neutrophil function is not clear, but various suggestions that have been put forward include a) inhibition of membrane calcium release [365]; b) blockage of membrane thiol groups [366]; c) inhibition of NADPH oxidase activity [366]; d) increasing cAMP levels [367] and e)

direct ROS scavengers, e.g. by making Cu complexes act catalytically to eliminate $O_2^{\bullet -}$ [368]. Evidence for such an action comes from i) the isolation of indomethacin-Cu complexes, ii) their inhibition of SF degradation induced by ROS generated enzymes [369], iii) reduced ROS products in serum and SF in RA-NSAID [100], iv) inhibition of bacteriocidal action of OH^{\bullet} by NSAID, v) pulse radiolysis showing NSAID react rapidly with OH^{\bullet} [370]. However, the in vitro evidence for these hypotheses remains questionable in view of the fact that levels of NSAID used were frequently unphysiological and in fact some of the inhibition could even have been artifactual, e.g. due to inhibition of receptor-ligand binding in the case of FMLP with

certain drugs.

90% of the serum NSAID's levels are normally protein-bound, but fortunately less so in SF, making effective levels higher here where it is most needed [371].

The main problem using NSAID's are those of aspirin hypersensitivity and more importantly the toxic side effects that these drugs have in ^{the} gastro-intestinal tract causing bleeding due to reduction of PG levels which are known to afford cytoprotection [372].

Because of the enormous variety of NSAIDS now available, the physician has a great choice and can keep changing the NSAID administred until he finds one which is most effective with least side effects for his patient.

In view of the fact that no RA patients could be found on no treatment at all and the least chemotherapy was NSAID treatment alone, such patients served to represent as near as possible the "RA disease" in this project.

3) Corticosteroids

In subjects whose disease activity is not adequately controlled by NSAID treatment, more potent and longer-lasting antiinflammatory drugs are employed: these are the corticosteroids in the form of hydrocortisone or prednisolone, which inhibit the generation of all

amino acid derivatives by preventing activation of phospholipase - A_2 (PL A_2). They are usually administered by intra-articular injection into the most inflamed joints often with startling and relative long-lasting effects.

This inhibition is thought to occur through the generation of a family of anti-phospholipase A_2 proteins, called lipocortins, of 32-40 kda, which are believed to block the active site on the enzyme or reduce the availability of its substrate. The mechanism of the anti-inflammatory effects of the corticosteroids via lipocortins - especially lipocortin 40 kda - include the following: a) inhibition of ROS production; b) inhibition of EA-rosetting via Fc receptors, but not Fc receptor expression; c) inhibition of the binding of IL-2 to its receptor [373] and as a consequence of this cause: inhibition of IL-2 production (since IL-2 - receptor interactions upregulate IL-2 production), inhibition of CD25 induction and therefore high affinity receptor production (since IL-2 is known to upregulate CD25 expression), inhibition of CD25 expression due to direct binding to the TAC antigen, and therefore inhibition of T cell proliferation in response to IL-2 or mitogens.

Corticosteroids are known to affect the distribution of T cell subsets with immunoregulatory properties, causing transient $T\mu$ lymphopenia ($T\mu_{\downarrow}$) and also $T\gamma^{\uparrow}$ [374,375], sometimes causing decreased T cell and monocyte numbers; they can also influence several in vitro tests for both spontaneous (naturally occurring) or mitogen induced

suppressor cell activity [376-378], so can be regarded also as immunosuppressive agents.

Because of their known immunosuppressive effect, RA patients on corticosteroids were avoided in this project.

Long-term use of corticosteroids is dangerous due to their immunosuppressive effects, peptic ulcer formation, adrenal suppression, development of thin and fragile skin, delayed wound healing and the likelihood of anti-lipocortin production, thus resulting in larger doses for equal efficacy on subsequent occasions when such treatment is needed. However, if it can be shown that such Ab's are only produced following certain steroids or certain routes (e.g. oral versus intravenous) - one could administer those steroids, or via a route with least likelihood of inducing Ab's. Some RA patients may already have anti-lipocortin Ab's in their blood and this may be pathological in its own right.

4) Second line drugs

Where disease activity remains unchecked by NSAID and/or corticosteroids, second line treatment is introduced. These drugs are referred to as "slow-acting agents", because their effects may not be apparent until after 4-6 months of continuous treatment. Because they appear to be able to halt or slow down the tissue destruction by altering immune reactivity, they are also termed "antirheumatic" or "disease remitting" drugs, but are better described as "disease

suppressing" or "modifying" drugs, since total clinical remission with them is extremely rare. They can reverse some of the damage of acute and chronic synovitis but not that seen in burnt-out disease. Improvement in clinical status can be assessed radiologically and by clinical indices, e.g. Ritchie index and laboratory parameters, e.g. ESR, CRP and RF levels.

This group of drugs includes the sulphydrate drugs, such as D-penicillamine and gold mercaptides, and drugs converted to sulphydrate metabolites in vivo, e.g. levamisole, it also includes the anti-malarial drugs, namely chloroquine, hydroxychloroquine and sulphasalazine. These drugs lack any direct antiinflammatory or analgesic or immunosuppressive properties, but are the only drugs to date known to halt the disease process, and as such they halt the inflammatory process too, which remains quiescent for some time after discontinuation of treatment but returns again eventually. Some of their in vivo effects and adverse side effects are shown in table 1.4. One other possible mode of action of sulphydrate drugs is by increasing vascular permeability and creating a histodilution barrier, diluting out the damaging agents in the joints, as can occur with the use of such agents in the stomach [378b].

Treatment with these drugs is hampered by the manifestation of toxic side effects; some non-lethal but annoying, e.g. skin rashes, loss of taste and other potentially life threatening effects, e.g. thrombocytopenia and neutropenia. These effects have limited their

TABLE 1.4

THE ACTION AND ADVERSE SIDE EFFECTS OF ANTIRHEUMATIC DRUGS

2 nd LINE DRUGS	ACTION (most data derived from in vitro studies)	ADVERSE SIDE EFFECTS
<p>CHRYSOTHERAPY:</p> <p>Oral Auranofin.</p> <p>Intravenous sodium aurothiomalate or sodium aurothioglucose, 2t</p> <p>10-20 mg/week or 50 mg/2-3 weeks or 200 mg/month.</p> <p>Serum levels 1 µg/ml</p>	<p>↓ PMN function especially ROS production, phagocytosis and chemotaxis</p> <p>↓ C' cascade</p> <p>↓ in vitro L₀ responses, normalises in vivo L₀ responses, inhibits lysosomal enzymes. Au may inhibit Mϕ function and could block SH groups, but thiomalate may reduce oxidised SH groups</p>	<p>Agranulocytosis</p> <p>Proteinurea</p> <p>Thrombocytopenia</p> <p>Type I hypersensitivity</p> <p>Aplastic anaemia</p> <p>Rash</p> <p>Bad breath</p> <p>Yellow skin</p> <p>More toxic the lower the sulphoxidation (S-S) status</p>
<p>LEVAMISOLE $\xrightarrow{\text{in vivo}}$ OMPI</p>	<p>Protect α_1 proteinase inhibitor from ROS damage</p>	<p>Nausea</p> <p>Rash</p> <p>Loss of taste</p>
<p>D-PEN 125-750 mg/day starting with low dose and increasing intake with spaced increments.</p> <p>375 mg/day maintenance dose.</p> <p>Serum levels 10-50 µM</p>	<p>Inhibits T_H activity in presence of CuSO₄ by production of H₂O₂.</p> <p>Scavenges ROS, inhibits IgG aggregation, normalises Mϕ function, chelates Cu and Fe, reduces oxidised SH groups.</p> <p>Stabilises collagen, inhibits collagenase and MPO activity</p>	<p>Thrombocytopenia</p> <p>Proteinurea</p> <p>Rash</p> <p>Neutropenia</p> <p>Induction of SLE</p> <p>Blood dyscrasis</p> <p>Pemphigus</p> <p>Loss of taste</p>
ANTIMALARIALS	<p>Lysosomatropic effects</p> <p>↓ collagen degradation</p> <p>↓ IL-1 production</p>	<p>Retinopathy</p>
SULPHASALAZINE	<p>↓ acute phase response</p> <p>Possibly ↓ IL-1 production</p>	<p>Thrombocytopenia</p> <p>Nausea / vomiting</p> <p>Oligospermia</p>

wide-spread use and result in the necessitation of strict monitoring in those patients in which such treatment is deemed useful. Fortunately the demonstration of toxic side effects or inefficacy using one does not mean that similar toxicity and inefficacy is found with another, so the physician has the opportunity to try various treatments until the most effective with least side effects is found for the individual concerned. Because of their slow-acting nature, patience is required on the part of the doctor and patient in order to see if the drug is efficacious or not (see sulphhydrate treatment in later section).

5) Third line drugs

Where disease activity is not cured by the use of even second line drugs, third line agents are employed, as a final attempt to pacify the aggressive disease process. These drugs are cytotoxic/antimetabolic, e.g. azathioprine, methotrexate, cyclophosphamide and chlorambucil. These drugs are not directly anti-inflammatory or antirheumatic, but simply halt the disease by preventing dividing cells from replicating their DNA or by depleting the cells of DNA precursors. They are used to try and inhibit CD4⁺ T-cell and B cell hyperactivity and therefore inhibit the unchecked immune response. However, since these drugs are not selective for activated lymphocytes, but inhibit all actively dividing cells in the body, e.g. those of the GI tract, bone marrow and gonads, they result,

after long-term use, in obvious risks of bleeding, toxicity, immunosuppression and sterility, which seriously restrict their administration. Sometimes they are given in low doses in order to reduce the amount of corticosteroids that need be given, since a potent combination of low dose corticosteroid and low third line treatment is thought less harmful than high doses of either alone.

When all such previous treatments have proved inefficacious, the following treatments are also possible: a) the administration of lipid-soluble cyclosporin-A as an immunosuppressive agent via its ability to inhibit IL-2 receptor expression and therefore cell proliferation; b) the administration of Campath-6 which is a cytotoxic antibody to IL-2 receptor bearing cells. It causes short-lived therapeutic benefits, but cannot be used for long in case antibodies are formed against this rat protein.

PATIENT MANAGEMENT

Because RA patients have such a variable clinical course, each patient must be assessed thoroughly and individually including joint assessment (number and severity), patient grip, changing blood picture, e.g. ESR, Hb, acute phase proteins and RF, functional disability, radiographic analysis. On the basis of all these measurements, a plan of management is drawn up, including physiotherapy, - programs of rest and exercise, occupational therapy and drug therapy. To prevent further joint destruction synovectomy may

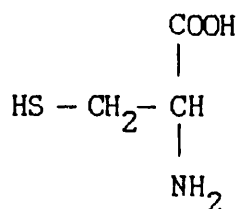
be carried out early in the disease, or sometimes late in the disease when the joint has already been destroyed. The joints that have been destroyed by erosive disease can be replaced with synthetic prostheses (e.g. hip, knee and metapthalangeal joints).

THE ROLE OF THE SH GROUP IN RA

a) The biological importance of sulphydryl groups

The SH group is one of the most ubiquitous ligands in biological systems, it is also one of the most reactive, taking part in several biochemical and metabolic processes, particularly those involving "SH-S-S exchange reactions". The ubiquitous free SH or S-S bonds are involved in the following: 1) structure and function of membranes; 2) enzyme systems - important in activation and/or substrate binding -, e.g. adenylyl cyclase; 3) membrane transport systems, e.g. Na^+/K^+ specific ATP-ase, Ca^{2+} channels; 4) hormone action and receptors, e.g. S-S-linked adrenaline receptors; 5) conformation of protein structure; 6) metal sequestration; 7) cell division; 8) recognition systems, e.g. S-S linkages in CD8, TCR, CD28 and CD11 (C3bi) antigens and SH in FCR function, and glucocorticoid receptor binding; 9) SH important in vascular integrity, blood clotting, cell migration, ROS scavenging and membrane attachment; 10) inflammation; 11) immune reactions; [379,380].

Free SH groups on enzymes, serum proteins or membranes are the result of the aminoacid cysteine:



The SH group in this molecule can covalently bind to small MW molecules to form glutathione, or larger ones to form macromolecules. Oxidative combination of two free cysteines results in cystine, and if two cysteine residues are close together in the same protein, they could react to form intramolecular S-S bonds or, if close to ones in a separate molecule, intermolecular bonds. The ubiquitous nature of SH and S-S groups makes them the ideal candidate to be responsible for diseased states, since simple blockade or oxidation of the SH group, or incorrect positioning of S-S bonds or reduction of S-S bonds could obviously have disastrous consequences.

It has been suggested that the autoimmune disease, e.g. RA, should be called the "auto-disulphide-dysynthesis diseases" since they often seem to display reduced free SH, increased S-S levels and abnormal proteins due to incorrectly paired S-S bonds, and therefore altered tertiary structure, resulting in abnormal biological activity, e.g. alterations in structural proteins, enzymic activity, surface antigens and receptors.

An intracellular mechanism for regulating cell surface sulphhydryl-groups in the form of glutathione and its associated enzymes has been postulated [381,382], and of course if this is inadequate it could lead to a diseased state. The biological processes in which these functional groups are involved of most relevance to RA are inflammation [383] and the immune response [384,385]. Regarding the immune response, SH groups are important in lymphoid cell proliferation [384,386] and differentiation [387], Ab production [388], Fc receptor function [389], and monocyte and lymphocyte interactions for Ab synthesis [289]. Since SH groups are important in all these processes, it justifies their investigation in a chronic autoimmune disease such as RA. It can be visualised that low molecular weight -SH containing molecules are very reactive, since the SH group is easily accessible. High molecular weight sulphhydrate enzymes are fairly reactive, but high molecular weight membrane sulphhydrate proteins are less reactive, due to their geographical arrangement and the reactive groups around them; thus some membrane SH groups are highly reactive, slowly reactive or totally masked. Factors assumed to play a role in determining the reactivity of a particular SH group in a protein are: 1) the nature of adjacent groups (particularly electrophilic ones); 2) electrostatic effects; 3) pH; 4) steric factors; 5) internal interactions of SH groups with hydrogen or S-S bonds [389b]. In trying to establish a role for SH or S-S bonds in various biological processes, a variety of reagents can be

used (see Table 1.5 and 1.6). A role for a thiol group in an isolated molecule or enzyme is quite easy to illustrate, but is more difficult in membrane proteins due to: a) lack of specificity of many of the reagents (i.e. react with other functional groups apart from SH); b) different reagents react with different SH groups, some reagents react with all SH groups, even—those not involved in specific cellular function; c) difficulties in distinguishing a primary effect from an effect secondary to inhibition of cellular metabolism, and finally: e) it is difficult to postulate a role for a thiol group in an unknown protein of unknown geographical arrangement that functions in an unknown way. Some of the SH reacting reagents are non-penetrating (e.g. PHMPA), slowly (e.g. NEM) or rapidly (e.g. diamide) penetrating.

Therefore, in order to test the significance of a membrane sulphydryl group in a particular cellular function, it is obviously necessary to find a reagent which is non-penetrating (hydrophilic and lipophobic) and with a high specificity for SH groups, particularly those that are functionally important. Of all the reagents shown, PHMPA best fulfills these criteria, thus it was used in this project as a blocking agent for free SH groups, to see which cellular functions are inhibited after their blockade. It should be noted that not all SH groups are functionally important; nor are all functionally important SH groups oxidised in RA; nor are all functionally important SH groups blocked by PHMPA; nor are all SH groups, which are blocked

TABLE 1.5

A WIDE RANGE OF AGENTS CAN BE USED TO INVESTIGATE THE IMPORTANCE OF SH GROUPS IN PROTEIN STRUCTURE AND FUNCTION AND ARE CLASSIFIED ACCORDING TO THE NATURE OF THE INTERACTION.

(Equations shown where relevant to the project)

Class of reagent	Interaction	Typical agents
1	"SH — S-S" exchange reaction e.g. oxidation of reduced SH groups $Q-SH + R-S-S-R \longrightarrow Q-S-S-R + R-SH$	L-cystine Oxidised (GSSG) glutathione Cystamine
2	Oxidation (dehydrogenation) $2Q-SH \xrightarrow{[O]} Q-S-S-Q$	γ -IFN ROS O_2 + Fe salts D-PEN + $CuSO_4$ Diamide Diethylmaleate (DEM) O-Phenanthroline-cupric complex
3	Blockade by mercaptide formation with heavy metals, e.g. $Q-SH + Hg \xrightarrow{H^+} R-S-Hg$	Hg and Au salts Organic mercurials e.g. PCMB (cell penetrating), PCMBSA & PHMPSA (non penetrating)
4	Alkylation	Iodoacetamide Iodoacetate Thiomersal
5	Addition to " = "	N-ethylmaleimide (NEM)
6	Acylation	Acid anhydrides
7	Arylation	Fluoronitrobenzene Trinitrobenzene Sulfonic acid
8	Raised pH	Alkaline agents

N.B. Fluorescent thiol interacting agents (Bimanes) can be used to directly indicate the position of internal SH groups (using mBBBr) or surface SH groups (using qBBBr).

TABLE 1.6

A WIDE RANGE OF AGENTS CAN BE USED TO INVESTIGATE THE IMPORTANCE OF "S-S BONDS" IN PROTEIN STRUCTURE AND FUNCTION AND ARE CLASSIFIED ACCORDING TO THE NATURE OF THE INTERACTION.

(Equations given where relevant to the project)

Class of reagent	Interaction	Typical agents
1	<p>"S-S — SH" exchange reaction</p> <p>e.g. reduction of oxidised SH groups</p> $Q-S-S-Q + 2R-SH \longrightarrow 2Q-SH + R-S-S-R$ <p>e.g. reduction of blocked SH groups</p> $Q-S-X + R-SH \longrightarrow Q-SH + R-S-X$	<p>Thiomalate</p> <p>2-ME</p> <p>Cysteamine</p> <p>phosphate</p> <p>L-cysteine</p> <p>Reduced (GSH)</p> <p>glutathione</p>
2	Oxidation	Performic acid
3	Blockade by mercaptide formation	<p>Dithiothreitol</p> <p>(DTT)</p> <p>Dithioerythritol</p> <p>Diethyldithiol-</p> <p>carbamate</p>

N.B. Redox potentials in order of decreasing effectiveness:

DTT > 2-ME > GSH > L-cysteine

in RA, functionally important.

Also it must be remembered that PHMPA acts irreversibly by surface SH blockade, whereas in vivo the main SH interacting agents are ROS, which can penetrate membranes so can interact with both internal and external SH groups to form S-S bonds, whose formation is reversible if the cell contains sufficient detoxification mechanisms. 2-mercaptoethanol (2-ME) was used to test the reverse reaction, (i.e. reduction of membrane S-S to SH) on cell function, since 2-ME too is surface acting.

b) The role of the SH group in the immune
and inflammatory response

Several aspects of the immune response appear to involve SH groups, and the idea that ROS could cause their oxidation in vivo has been given credence by the finding that addition of H_2O_2 to serum, oxidises the SH group on albumin in a dose-dependent manner, which also occurred if activated neutrophils or monocytes were used instead. In the particular in vitro system used, none of the other ROS nor ceruloplasmin oxidase activity, but only H_2O_2 was found to have this effect [96].

Numerous studies have shown that simple low molecular weight thiols, e.g. 2-ME, can potentiate the function of many immunological cells in vitro, and show a wide range of immunomodulatory effects,

despite the short half-life of 2-ME in culture (5 to 9 hours) and its narrow range of stimulatory effects ($2-5 \times 10^{-5}$ M), being toxic at higher concentrations and inhibitory either side of this range. A review of the literature shows that 2-ME: 1) can stimulate Ab responses, e.g. RF and PFC or Ig production, since it is a polyclonal B cell activator; [385]; 2) can stimulate lymphocyte proliferation in response to T cell dependent mitogens or allogeneic MLR [384,386]; 3) can stimulate cytotoxic T cell responses [390-392]; 4) can stimulate the growth of B-lymphocyte colonies [393]; 5) is weakly mitogenic for B cells; 6) can enhance lymphocyte viability; 7) can synergize with endotoxins in the induction of a B cell mitogenic response; 8) is required for optimal primary in vitro humoral immune responses; 9) is a generally recognized additive to cultures to improve PBMNC immune responses; 10) is required to maintain free membrane SH groups on NK-like cells late in their cell cycle ($G1_a$ to S), but early in the cell cycle of lymphocytes ($G0$ to $G1_b$); 11) can cause reduction of radiation-induced SH oxidation (thought to be caused by ROS); 12) can react with cystine in media and increase its uptake by cells, where it is metabolized and released as glutathione and cysteine, and can function as a serum component activator by reacting with the methyl-thiol group of FCS to form methyl-thio-disulphide which can be taken up by the cells, thus altering intra- and extra-cellular SH/S-S ratio in cells; 13) can react with albumin to form mercaptoalbumin, which is more resistant to auto-oxidation than non-protein thiols, thus has a stabilizing role in the maintenance of the SH content of

media; 14) can inhibit arachidonic acid mediated platelet aggregation; 15) can substitute for macrophages in the PFC response of spleen-cells depleted of adherent cells [394], this is explicable in terms of macrophages having a non-specific helper function to T- and B cells in the form of generating SH compounds, e.g. cysteine and glutathione, to maintain the SH/S-S ratio in the environment.

Not all small MW SH containing molecules behave like 2-ME: some of their actions are similar and others are dissimilar, and it seems that their efficacy depends on their configuration, state of oxidation and location of constituent groups on the molecules, and not just on their redox potential. 2-ME is envisaged to engage in SH/S-S exchange reactions aided by its ability to form hydrogen bonds between its -OH moiety and the cell surface, stabilizing its presence in the vicinity of an S-S group.

Not all the reactions of sulphydrate reagents are stimulatory; a few papers described inhibitory phenomena, e.g. 1) high concentration ($> 100 \mu\text{M}$) of sulphydrate reagents (D-PEN, glutathione, DTT, L-cysteine) were shown to be initially stimulatory for lymphocyte proliferation by assisting the conversion of L-cystine to L-cysteine, which is more readily taken-up by the cell, but later, due to the formation of mixed disulphides with L-cystine (which cannot be taken-up by the cell), they eventually deprived the cell of this essential aminoacid, thus causing inhibition [395]; 2) low molecular SH compounds were found to damage certain S-S linked receptors, e.g.

C3bi for EA-rosette formation and immunoadherence [396] on erythrocytes, lymphocytes and phagocytes, and 2-ME has been shown to disrupt the CD8, TCR, CD28, adrenaline and insulin receptors causing 2 bands on SDS electrophoresis [397]. Despite glutathione, DTT and L-cysteine all disrupting the C3bi receptor, only L-cysteine inhibited adrenaline responses in lymphocytes; thus again differences in SH reagent structures and steric hindrances affect sulphhydryl action [398]; 3) there is evidence that SH reagents can disrupt certain S-S soluble antigen specific MHC restricted factors, e.g. T_H factor [398b] and T_S factor [398c].

Using a variety of SH reacting agents (e.g. the oxidising agent diamide, blocking agents PHMPSA, NEM and DTNB, and the alkylating agent iodoacetamide [399]), SH groups were shown to be involved in early events in lymphocyte activation (G0 to S). The early events in the cell cycle where SH interacting agents may interact are: inhibition of the hexose-monophosphate shunt (which increases glutathione levels), inhibition of Na^+/K^+ ATP-ase (needed in lymphocyte transformation), alteration in Ca^{2+} fluxes and in membrane permeability, inhibition of adenylyl-cyclase activity (cAMP is an important regulator of lymphocyte metabolism, immunological reactivity and tolerance), inhibition of receptor binding and function, inhibition of phospholipase A_2 activation and its subsequent actions on aminoacid metabolites, oxidative metabolism and phosphorylations of enzymes and receptors, inhibition of tubulin polymerization (important

in α -amino-isobutyric acid transport and DNA synthesis). Some of the events that could be affected later in the cell cycle are the consequences of some of the events listed above, e.g. 1) inhibition of induction of ribosomal associated protein kinase that phosphorylates eif-2, blocking initiation of protein synthesis (due to a rise in G S S G), and 2) inhibition of enzymes required for RNA and DNA synthesis. The different time-courses of inhibition shown by the various SH reacting molecules, do not necessarily imply action on unique sets of SH groups, but simply reflect their different half-lives in cultures and the fact that some are rapidly penetrating (diamide) but others are not (PHMPSA).

Several lines of evidence suggest that the important SH groups for lymphocyte activation are on the cell surfaces and not intracellular, e.g.: 1) both diamide and PHMPSA inhibit lymphocyte responses, yet the former enters the cells fast [399] and the latter is not penetrating [400]; 2) glutathione and 2-ME enhance lymphocyte proliferation, but cysteamine phosphate has no effect, yet the former are impermeant thiols and the latter is permeable and can be converted to a SH compound intracellularly [401].

c) The role of sulhydrylate-drugs in the treatment of RA

The fact that only the second line drugs, most of which are sulhydrylate drugs or are converted to thiol-containing metabolites in vivo, halt the disease process, would suggest that their effectiveness

is due to their SH groups which can be used to correct membrane and serum thiol levels by SH-S-S exchange reactions [402]. Raised serum thiol levels after sulphydrate treatment probably result from normalization of albumin levels due to the disease remission and by release of free SH groups that were previously locked in macroglobulins such as RF. Certain antiphlogistic agents (steroids and NSAID) can also sometimes normalise defective SH levels, but do so probably by influencing exposure, reactivity or number of SH groups or by accelerating SH-S-S exchange reactions, so function via different mechanisms [403].

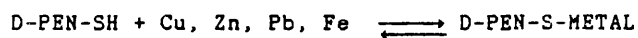
The sulphydrate drugs should theoretically be able to rereduce oxidised intracellular thiols (e.g. GSSG), extracellular soluble (e.g. albumin and cysteine) and cell surface thiols (e.g. involved in ligand receptor binding or receptor-G protein interactions) and could therefore have a common mechanism of action. With respect to D-PEN, possible modes of action include those shown in table 1.7. Such sulphydrate drugs include D-penicillamine, gold-thiol complexes (aurothiolglucose, aurothiolmalate, metabolised to Au and thioglucose or thiolmalate in vivo), auranofin, the immunopotentiating drug levamisole which also has an antioxidant effect and a thiol metabolite in the form of OMPI, and the antioxidants such as the vitamin B₆ derivative 5-thiolpyridoxine (5-TP), tiopronin (also called Thiola or 2-Mercaptopropionyl glycine) and diethyl dithiocarbamate (also called DDC or imuthiol, see Fig. 1.4). However, chrysotherapy and D-PEN

TABLE 1.7

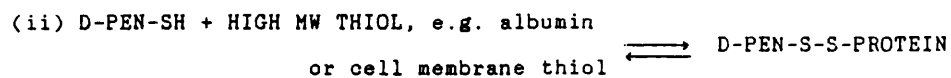
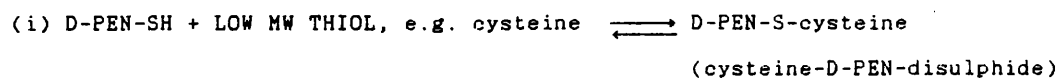
POSSIBLE BIOCHEMICAL INTERACTIONS OF SULPHYDRATE DRUGS RELEVANT TO RA.

(Using D-PEN as an example).

COMPLEX FORMATION WITH HEAVY METALS



DISULPHIDE FORMATION



THIOL DISULPHIDE EXCHANGE REACTION

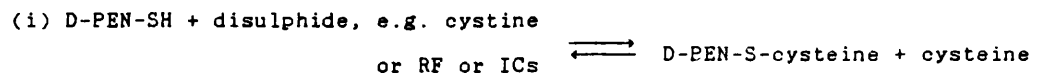
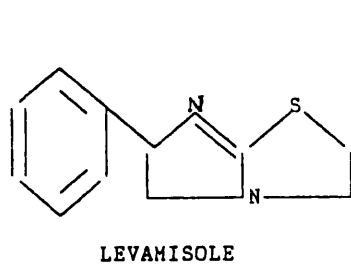
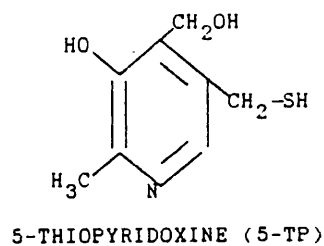
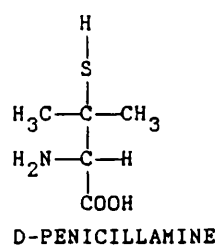
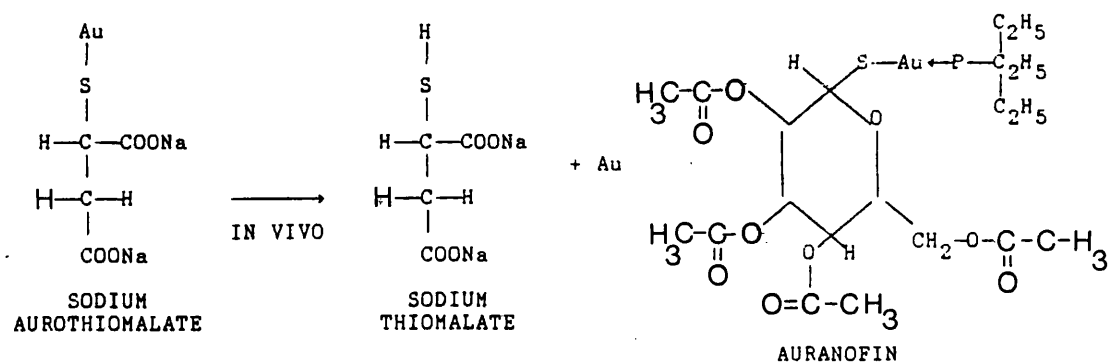
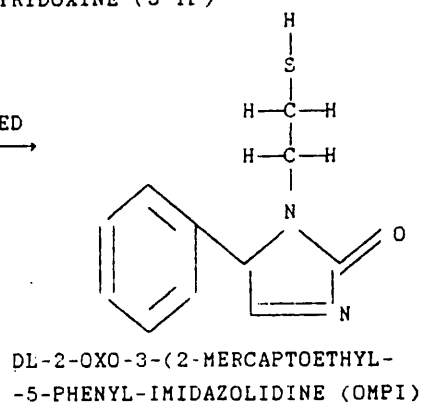


FIGURE 1.4

CHEMICAL STRUCTURES OF SOME SECOND-LINE ANTIRHEUMATIC DRUGS AND THEIR METABOLITES



$\xrightarrow[\text{IN VIVO}]{\text{METABOLISED}}$



treatment form the main sulhydate drugs in RA. The injectable gold salts and D-PEN are water soluble, so probably preferentially affect extracellular and surface thiols, but aurothioglucose being lipid soluble probably preferentially affects intracellular thiols, however all have been shown to eventually normalise serum and ^{soluble and} SF_A membrane thiols [404,405] and intracellular thiols [406,407].

Following sulhydate drug treatment, serum thiol expression returns to normal in good clinical responders [404,405]. It was found that total serum thiol levels in RA are low due to reduction in "slow-reacting thiol groups" in preference to fast-reacting groups, and the sulphydate drugs tend to raise fast-reacting SH groups rather than the slow ones, so although overall SH concentrations are normalised, their reactivities are not [408]. This is perhaps one reason for their ineffectiveness in some people and their inability to cure the disease.

RA patients on the disease remitting sulphydate drugs (D-PEN and aurothiomalate) were used in this project to represent "inactive RA disease". Sulhydate treatment being the in vivo equivalent of 2-ME treatment in vitro, known to improve immune responses in normals and particularly RA's due to increasing surface membrane thiol levels.

However not all thiol compounds are antirheumatic drugs, e.g. cysteine is inefficacious due to its unstable thiol group, so the bioavailability of the SH group is important with respect to their

effectiveness. Thus, when considering novel thiol compounds, thought must be given to the metabolism of the drug and compounds with structural arrangements likely to protect thiol groups from metabolic pathways should be favoured since their effectiveness depends on the stability of the SH group.

d) Serum and membrane SH levels in RA

i) Serum thiols in RA

Depressed serum thiol levels are a feature of active RA and other connective tissue diseases, e.g. SLE, which have disturbances in the immune system and are inflammatory conditions, but normal or low SH levels are found in inflammatory conditions showing slight or no immune system disturbances, e.g. AS, PSA and OA respectively [409]. Inflammation is present in all these conditions, but is more severe in RA and SLE, so this is indirect evidence for an association with ROS involvement.

It has been found that serum SH levels are the same in RA patients whatever the sex and whether sero-negative or positive and that serum thiols fall with increasing age irrespective of the RF state of the disease [410], however others have found decreased serum SH levels occurred in conjunction with increased RF levels and that patients with connective tissue diseases plus vasculitis have more severely reduced thiol levels [409]. This inverse correlation between

serum thiols and age is not an RA specific phenomenon, but true also of healthy subjects greater than about 70 years [411] and it has been suggested that aging was caused by increased presence of ROS causing reduced -SH levels and that life could be prolonged by diets rich in SH groups [412].

Serum thiols do not characterise any special target molecule, although the main molecule measured is albumin (which comprises 85-90% of the SH groups measured, the rest being within the globulin fraction), but merely reflects the redox potential of the serum. Normal albumin thiol concentration is about 0.7 thiols per molecule [413], the reason for this non integral number arises because some thiols form disulphide bonds with small SH compounds, e.g. cysteine to form mixed disulphides. The consequences of albumin oxidation are unknown, as no specific function has been attributed to their SH groups. Serial measurements of serum SH levels in RA showed them to correlate inversely with disease activity [410].

Serum thiol levels are therefore a good marker for persistent synovitis and a useful index of chronic inflammation, reflecting enhanced phagocytic activity [414].

It must be remembered that reduced thiol levels are also observed in other disease states, e.g. cancer and leukaemia, so are not disease specific but could be better described as a marker for lymphocyte activation, the lymphoblasts in RA originating from the main

inflammatory site, i.e. SM [415].

Reasons for depressed serum thiol levels in RA

1) As a result of further oxidation and subsequent mixed disulphide formation of albumin [416].

2) During acute inflammation there is a general alteration of liver protein synthesis from albumin production to production of the acute phase proteins (APP's), hence albumin synthesis falls in order to allow for the increased synthesis of APP's important in host defence [417]. However reduced albumin levels can't be the only reason for depressed serum thiol levels since the two do not correlate [405].

3) One of the APP's is caeruoplasmin and it has the ability to rapidly oxidise low molecular weight thiols, e.g. cysteine and thiolmalate to disulphides [418]. Steric hindrance prevents its direct interaction with albumin, however its disulphide products can result in SH-S-S exchange reactions with SH groups on albumin [413] and it could therefore be an indirect determining factor in reduced serum thiol levels.

ii) Cell surface thiols in RA

Reduced cell surface thiols have also been found on erythrocytes [431], neutrophils [419], monocytes and lymphocytes in RA [289].

Reasons for depressed membrane thiol levels

1) It has been proposed that reduced membrane SH levels in RA reflect an attempt by the body to scavenge ROS produced in inflammatory reactions [414].

2) Because an equilibrium exists between membrane thiols and serum thiols, defects will be seen in both the compartments.

Thus, the alteration in SH/S-S status in RA is systemic, occurring in many different biochemical compartments, e.g. intracellularly (GSSG[↑], % of S-S on Hb[↑]) and extracellularly in the form of oxidation of thiol groups on α_2 , β and γ globulins leading to RF formation and % of S-S in albumin [↑] and reduced cell surface SH levels. Synovial fluid SH levels are also low in RA but strangely to an equal extent as the serum, despite the increased ROS levels to be expected here, and this finding has been proposed to be due to the rapid exchange of proteins between the SF and blood or that SF contains more factor(s) which protect protein thiols [420]. In all cases the reduction in thiol levels wherever it occurs is due to direct oxidation of SH groups to S-S bonds by an association with other molecules or cells possessing thiol groups. Since the thermodynamics of the reaction $2\text{RSH} + \text{O}_2 \rightleftharpoons \text{R-S-S-R} + \text{H}_2\text{O}_2$ indicates that it is an energy requiring process to maintain the reduced thiol state, it has been postulated that perhaps a biochemical disturbance exists in RA that adversely affects the equilibrium making it shift

spontaneously to S-S formation, which could account for the formation of the macroglobulins like RF [409]. Since even antiphlogistic drugs accelerate SH-S-S exchange reaction and even in the absence of drugs serum thiols can normalise if disease activity diminishes [404], but only sulphhydrate drugs specifically alter the disease course and only they are truly immunoregulatory, it suggests that sulphhydrate drugs have their specific action not on serum thiols but possibly surface SH groups on cells associated with chronic inflammation, namely lymphocytes, monocytes [404] and neutrophils.

The following is a review of the importance of free SH groups for monocyte and neutrophil function relevant to RA:

It has been found that PBMNC from RA-NSAID show defective PWM stimulated IgG production which can be normalised by 2-ME treatment, but patients on D-PEN show normal IgG production and 2-ME does not enhance this effect [289]. It has been shown that monocytes require free cell surface SH groups for their accessory function in the immune response and that this is impaired in RA due to SH oxidation. The same authors found free cell surface SH groups on lymphocytes were also necessary for optimal IgG production, however the ability of lymphocytes to recognize antigens or mitogens presented by monocytes to them is not thiol-dependent [289]. Further support for the concept of the importance of free cell surface SH groups in cell function comes from studies showing 2-ME enhances PHA stimulated PMNC proliferation of RA cells [86]. In trying to delineate monocytic

functions that are dependent on SH groups relevant to their accessory function, it has been found that antigen presentation, calcium mobilization and altered aminoacid levels are not thiol-dependent mechanisms, by using PHMPSA [86], however in contrast to this work $\text{CuSO}_4 + \text{D-PEN}$, known to produce H_2O_2 , does inhibit cluster formation when cells are stimulated by PWM or PHA [421,422], perhaps reflecting the different mechanisms of action of PHMPSA and H_2O_2 . In more recent work in our laboratory, PHMPSA has been shown to affect membrane fluidity and calcium mobilization (N. Hall, personal communication).

According to Hewlett's hypothesis (personal communication), macrophages activate albumin (promaSF) to mercaptalbumin (maSF), which is then an essential serum factor for lymphocyte activation, it can be seen that reduced macrophage membrane SH groups or reduced levels of small molecular weight sulphhydrate products from them could inhibit maSF formation and thus contribute to abnormalities in the immune response in RA disease. However this theory is unlikely in view of the observation that, although albumin is essential for lymphocyte growth in culture [289] and an immune response, possession of free serum SH groups on albumin is not necessary [289].

The role of surface SH groups in monocyte/neutrophil oxidative metabolism is extremely controversial. Some authors claim that PHMPSA has no effect on any neutrophil function [423], whereas others suggest that whilst PHMPSA has no effect on $\text{O}_2^{\bullet -}$ production, it does inhibit H_2O_2 production [427,428], suggesting an SH-dependent pathway for H_2O_2

production following some stimuli and the two pathways being independent, in agreement with another author [425]. In this latter work increased background H_2O_2 but not $\text{O}_2^{\bullet-}$ production in RA was found, suggesting perhaps an SH-dependent pathway for $\text{O}_2^{\bullet-}$ production independent of H_2O_2 production [425,426], and suggesting that an H_2O_2 production pathway is preferentially activated in RA. Alternatively one could argue that the increased H_2O_2 production reflected an S-S-dependent pathway; evidence for this hypothesis comes from the observation of raised spontaneous ROS production by monocytes [86,289], and neutrophils [99] from RA-NSAID, suggesting that surface SH auto-oxidation raises ROS output and that S-S are important in ROS production. However, 2-ME treatment does not inhibit spontaneous H_2O_2 production [86]. In agreement with the SH-dependent $\text{O}_2^{\bullet-}$ proposed pathway, it has been shown that sulhydryl drugs lead to an increased rate of monocyte $\text{O}_2^{\bullet-}$ production after immunological stimulation (but not unstimulated or biochemically stimulated [425,426]) perhaps by affecting FCR receptors known to contain SH groups [389]. Further evidence to support the SH dependence of ROS production is the finding that PHMPA inhibits receptor-stimulated monocyte $\text{O}_2^{\bullet-}$ [86] and H_2O_2 [99] production, and neutrophil H_2O_2 production [99,427,428], and is substantiated by other work showing SH compounds increase subsequent receptor-stimulated $\text{O}_2^{\bullet-}$ release [419]. These reports suggest that both H_2O_2 and $\text{O}_2^{\bullet-}$ pathways may be -SH dependent. The fact that $\text{O}_2^{\bullet-}$ production from neutrophils appears not to be inhibited by PHMPA [427,428], but is from monocytes, suggest that perhaps different

pathways are used by the two cells. However, paradoxically, 2-ME treatment of RA NSAID monocytes does not raise background and stimulated H_2O_2 production, and RA D-PEN background and stimulated H_2O_2 production were found not to be significantly different to that from RA NSAID [86]. These findings suggest ROS production is independent of surface SH oxidation, which is very strange in the light of substantial evidence from in vivo and in vitro studies suggesting anti-oxidants and free radical traps are antiinflammatory, and protect neutrophils and monocytes from auto-oxidation. The conflicting data could be interpreted as PHMPSA inhibiting oxidative metabolism via blockade of SH groups through its Hg moiety, whilst rheumatoid cell surface oxidation in vivo is the result of disulphide formation with cysteine molecules [416]. Also, it is possible that Hg may react with SH groups involved in oxidative metabolism that are inaccessible to cysteine.

Whilst membrane penetrating SH group inhibitors (which inhibit extra and intra-cellular thiols, e.g. mercuric chloride and NEM) have been shown to inhibit neutrophil adhesiveness, motility, phagocytosis and $O_2^{\cdot-}$ production [423,428,429]. Surface SH group blockers inhibit the hexose monophosphate shunt [427].

It is possible that during periods of oxidative stress, where enhanced phagocytic activity is creating large amounts of ROS, as in RA, the neutrophils, monocytes and macrophages would be prone to inhibition. This auto-oxidation is probably a physiologically relevant

process to limit the neutrophil etc. response to a particular stimulus. In view of the reduced intra- and extra-cellular SH levels in RA (known to be ROS scavengers), and depressed ROS scavenging enzymes, one might expect ROS production to be a self-limiting process, but the rapid repletion and short half-life of neutrophils means that there is a constant source of potentially damaging cells, which is why in vitro studies frequently show RA neutrophils producing excess ROS when both unstimulated and stimulated. The less rapid repletion and longer half-life of monocytes and lymphocytes mean that the former may become less important than neutrophils in the inflammatory reaction, due to auto-oxidation, and that the latter will remain in the circulation but blocked for a considerable time, possibly resulting in the immune dysfunction seen. It is possible that enhanced background H_2O_2 production in RA monocytes causes the reduced IgG synthesis from, or proliferative response of, PBMNC observed in in vitro assays. However, some authors find that the addition of catalase does not improve the response, suggesting that this is not the mechanism involved [86]; in contrast others have found catalase does increase RA-NSAID lymphocyte proliferation compared to normals (Panayi G.S., personal communication).

Neither H_2O_2 nor $O_2^{\bullet -}$ are able to reverse PHMPA blocked IgG responses in normals, but H_2O_2 and $O_2^{\bullet -}$ were shown to inhibit mitogen stimulated Ig synthesis [86]. The discrepancy between one author's finding of no significant differences in H_2O_2 production from

unstimulated and FMLP stimulated monocytes from RA NSAID, compared to RA D-PEN [86] compared to another author's finding that immunologically stimulated monocytes from RA D-PEN produced more $O_2^{\bullet-}$ than RA NSAID or normals [425,426], could be explained in terms of the different stimuli used, since FMLP acts through receptors which are probably not affected by sulphhydrate treatment, but HAGG acts through SH containing Fc receptors. Another possibility is that there may have been disease activity differences in the RA population used.

One would imagine that if sulphhydrate treatment did enhance oxidative metabolism, then the inflammatory situation would worsen, but the fact that it does not - and in fact an amelioration occurs - suggests that they probably do not function in this way in vivo.

It has been shown using neutrophils that NADPH oxidase is dependent on free SH groups for its action [429b], and since it is a membrane associated molecule this could explain the auto-oxidation phenomena that occur.

Perhaps the inability of 2-ME to increase background monocyte H_2O_2 output in vitro [86], but the opposite finding from monocytes of RA-D-PEN compared to normals [86] and the finding that thiol compounds increase neutrophil $O_2^{\bullet-}$ production in vitro [419], suggest that different SH compounds may have varying affinities towards membrane SH groups, and so afford different levels of auto-oxidation protection.

There has been an interest in the role of serine proteases

(esterases) on neutrophil and mononuclear phagocytic function and using potent inhibitors and synthetic substrates of them, several studies have shown protease activity to be essential for $O_2^{\bullet-}$ production [430]. It has been suggested that the protease involved in $O_2^{\bullet-}$ production is a chymotrypsin or trypsin-like SH-containing molecule since the inhibitors used are known potent SH group inhibitors [430]; this is further substantiated by the fact that GSH prevents this inhibition [424].

It has been hypothesised that the hyperactivity of the inflammatory and immune response in RA could be explained by suggesting that macrophage-lymphocyte interactions initiated by an unknown antigen could be locked together in the oxidative conditions in the synovium by S-S bonds causing further ROS production with resultant uncontrolled inflammation and tissue destruction and that D-PEN treatment has an ameliorating effect by acting as a reducing agent to inhibit this process [289].

Because not all the reactions with PHMPA mimic those with H_2O_2 (because of their different mechanisms of action and the fact that scavenging enzymes destroy H_2O_2), attempts have been made to find a reversible SH blocking agent that more closely mimics the effect of H_2O_2 ; however none was found, so PHMPA remains the agent of choice [86]. The most promising of the reagents to date is O-phenanthroline cupric iron complex, an impermeant SH oxidising agent. However 2-ME only partially reverse its effect, whereas 2-ME can completely reverse

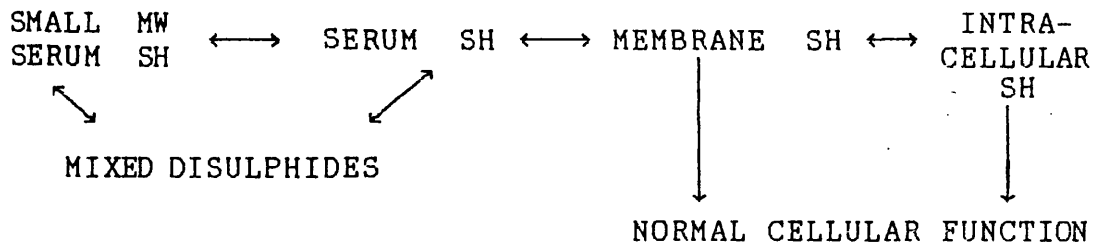
the effect of ROS [430b].

In serum or SF, where an equilibrium exists between albumin and low molecular weight thiols (usually cysteine, but sometimes GSH) producing mixed disulphide formation [416], it is possible to envisage that the equilibrium could extend to cell membrane thiols. It would seem reasonable, therefore, to suppose that whatever oxidises serum thiol groups could also oxidise cell surface thiols, and if permeable, intracellular thiols too. ROS ($^1\text{O}_2$, H_2O_2 , OH^\bullet and $\text{O}_2^{\bullet-}$) fulfill all these requirements. The possibility that this proposed SH-S-S equilibrium exists is substantiated by the finding that erythrocyte cell surface thiols are low in RA and correlate with low serum thiol levels [431], and by the finding of an association in normals between free SH groups on PBMNC and serum SH levels [289]. Therefore serum thiols are an indirect assessment of the SH status of cell membranes and since the methodology for their measurement is quicker and simpler and requires less blood, analysis of serum thiols was carried out in this project.

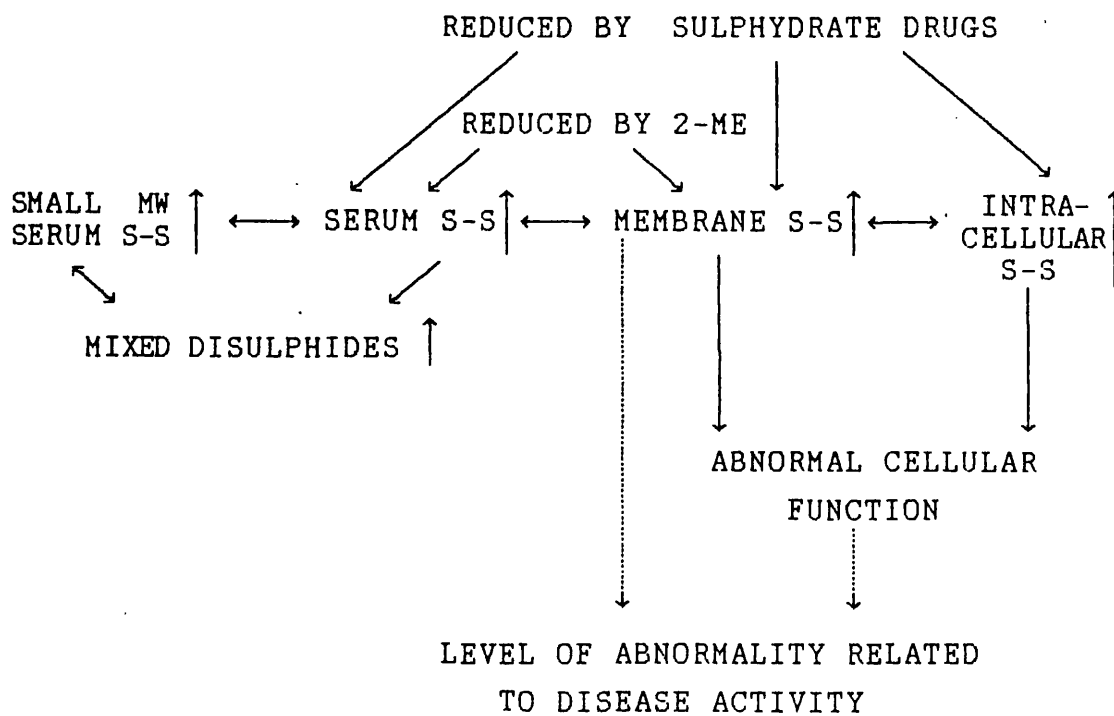
SH depression could lead to abnormal protein configuration, e.g. aggregation of IgG which could serve as an antigenic stimulus for an autoimmune phenomenon resulting in RF production. Depletion of SH groups in the local environment of immunocompetent cells, e.g. SM, could alter or disturb the normal function of lymphocytes residing

there. Reduced serum thiols could depress membrane thiols, which could reduce plasma and lysosomal membrane permeability causing release of intracellular constituents, e.g. lysosomal enzymes - which could initiate tissue damage and collagen release -, e.g. DNA - which along with collagen could act as antigens for autoantibody production - . Depressed thiols could reduce the activity of enzymes involved in maintaining a normal inflammatory and immune status and activate others, e.g. neutral collagenases, perpetuating inflammation. Decreased thiols could inhibit thiol-dependent receptors and perhaps stimulate S-S-linked receptors.

Thus on theoretical grounds SH depletion appears a plausible factor in both the induction and pathogenesis of RA (causing B cell hyperactivity, defective T cell mediated immunity and unchecked inflammation). One could envisage the following interactions between serum SH, membrane SH, disease and mode of action of sulphhyrate drugs (the scheme is shown for blood, but is equally applicable to other compartments, e.g. SF):

IN HEALTH:IN-RA:

IN OXIDATIVE CONDITIONS, e.g. \uparrow ROS:



The introduction would not be complete without a brief summary of other disease states used for comparative purposes in this project. AS/PSA are two of the spondarthritides which are inflammatory conditions with very slight or no disturbances in the immune system (but where present they contribute at least in part to the disease pathogenesis by translating the aetiological trigger(s) into disease manifestations), thus AS/PSA patients were used as "arthritic inflammatory controls".

ANKYLOSING SPONDYLITIS (AS)

AS is defined as a chronic disorder of the spine and the sacro-iliac joints, often with symmetrical involvement in which inflammatory lesions are associated with progressive stiffening of the spine and radiological calcification of spinal ligaments leading to a characteristic "bent posture". It is more common in males (males:females 5:1) and its aetiopathogenesis is probably a combination of genetic (being highly associated with HLA B27, present in > 95% patients) and environmental factors (e.g. trauma, and/or infectious agents like klebsiella). There is often a presence of MHC-I-restricted auto-Ab production to cartilage peptidoglycan. Peripheral joint involvement is also sometimes present (shoulders, hips, knees), but, unlike RA, it is non-destructive, thus it does not present with radiological changes as in RA. SF changes are "non-specific inflammatory" and similar to RA. There can be

musculo-skeletal and non-musculo-skeletal involvement; despite the tendency for a defective CON-A-induced suppression of T cell responses to be associated with HLA B27 (in JRA+, [268]), no evidence of a defect has ever been noted in AS, and, if anything, they are as good or better than normals [266], they also show normal IL-2 production and normal AMLR. Thus they can serve as a suitable disease control group for the CON-A induced suppressor cell assay, they also show normal serum SH levels [409], thus are a good control group for this assay too.

PSORIATIC ARTHRITIS (PSA)

PSA is defined as the association of psoriasis with inflammatory arthritis, usually in the absence of RF. The psoriasis can involve dermal and/or nail dystrophy. The associated arthritis can be peripheral (but unlike RA often only mono- or oligo-articular), and/or axial (spondylitis as in AS). When RA is present, it usually means that it is not PSA, but RA with psoriasis. PSA shows an equal sex ratio and is probably mediated by multifactorial inheritance, with a genetic influence (since it runs in families and has certain HLA associations) and environmental influences (e.g. trauma and/or infection).

In both AS and PSA, co-diseases include chronic inflammatory bowel disorders (e.g. ulcerative colitis) genitourinary tract infections, cardiac conduction defects and ocular inflammation

(usually iritis). The ESR is often raised and both groups are treated with topical steroids for eye involvement and NSAID to reduce inflammation. For severe peripheral arthritis in PSA, sulphhydrate drugs are sometimes used. The outlook for both diseases is however far better than for RA, probably due to the lesser immune system aberration.

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

SLE is a true connective tissue disease (so-called "collagen disease"). As its name implies, it is a disorder characterized by multisystem involvement (most organs and the musculo-articular system), and usually presents with a combination of the following signs: arthritis, rash, pleurisy, nephritis, neurological disease, unexplained fever and weight loss. Like RA, it is a disease of uncontrolled inflammatory processes and hyperactivity of B cell responses and defective T cell-mediated immunity, probably due to immuno-dysregulation, which is probably more aberrant than in RA, resulting in a more serious condition. The multisystem involvement seems to be mediated by ICs.

Like RA, it is an autoimmune disease, as evidenced by the presence of several auto-Ab (anti-dsDNA, anti-ssDNA, anti-dsRNA, anti-collagen, RF, anti-RO, anti-SM, anti-RNP, anti-La, anti-CD8, anti-erythrocyte membrane, anti-neuronal and anti-cardiolipin Abs). The anti-dsDNA Ab is thought to cross-react with glycosoamines,

anti-cardiolipin Ab predisposes to thrombosis and miscarriages. The anti-CD8 Ab probably cross-reacts with brain and trophoblastic cells, leading to cerebral damage and spontaneous abortions. The anti-erythrocyte membrane Ab leads to reduced C¹ receptor levels (e.g. C3b), preventing IC removal and the activation of the C¹ cascade, the use of C¹ in ICs leading to C¹ depletion.

Although the aetiology is not known, it seems to be the result of a combination of genetic (associated with HLA-A1, B8, DR3), infectious, autoimmune, environmental factors (possibly drug-induced, e.g. D-PEN), and even hormonal status (suggested by the female predominance). It may be that Ab's made against viral or bacterial polynucleotides in the course of a normal immune response, destined to eliminate the microbial Ag, unfortunately cross-reacted with many host cellular components forming ICs. The ICs cause C¹ cascade activation of both classical and alternative paths, and deposition of ICs (containing C¹ Ag and Ab) in tissues, resulting in inflammatory and immune reactions injurious to the host's cells, often in the kidney (nephritis) or skin (facial rash or alopecia vasculitis); alternatively there could be a persistent and self-perpetuating Ag leading to constant elevation of ICs. As in RA, the arthritis is symmetrical, but unlike RA, it is less severe in terms of deformities, and is non-erosive. There is often vascular involvement, probably the result of increased phagocytic activity due to IC stimulation, leading to lysosomal enzyme release and ROS formation, since raised background

H_2O_2 levels have been shown to correlate with factor VIII Rag levels, the latter being an indicator of microvascular damage [432]. The vascular involvement can lead to Raynaud's phenomenon and at the extreme, digital gangrene. SLE is more common in negros than in Caucasians, and usually presents itself at an age of < 40. If there is only skin and joint involvement, the prognosis is good; but is poor when renal or cerebral involvement occur (when early death is likely). The prognosis is worse than in RA, probably due to the more severe inflammatory and immune regulatory abnormalities.

Treatment involves sun cream barriers to avoid photosensitivity, NSAID for the arthritis, hydroxychloroquine for the systemic involvement and/or low dose steroids. In renal lupus high dose steroids or cytotoxic drugs are employed.

SLE represents the paradigm of an autoimmune disease known to be associated with gross and varied immunological abnormalities, and especially with defective number and function of suppressor cells. There is evidence in the literature for defective Ag non-specific suppressor activity in the form of CON-A induced suppression of T and B cell responses [433,434], defective spontaneous SLISA activity [439] and defective Ag-specific suppressor activity related to immune responses to DNA [435] or ovalbumin [436]. On the other hand, SLE involves defective IL-2 production, possibly as the result of an excess number or potency or increased PGE_2 sensitivity of radiosensitive T8 and non-T spontaneous suppressor cells, since

removal of spontaneous suppressor cells corrects IL-2 production [435b]. The defective IL-2 production could also account for defective AMLR and defective proliferative responses, since exogenous IL-2 normalises both [435c]. SLE may also involve excess spontaneous adherent suppressor cells which are probably macrophages. The defective CON-A induced suppressor cell activity has been shown in some cases to be due to the presence of anti-T8 Ab, and in others by excess spontaneously active naturally occurring suppressor cells. It is also possible that the defect could be due to viral ablation of suppressor cells or that B cell hyperactivity results from their direct activation by viruses. Other studies have suggested defective suppressor activity may be an artifact caused by dilution of normally functioning T_S by excess non-suppressor cells [437]. Still others have shown no defective suppressor activity but defective helper activity [436b], in contrast, others have shown hyperactive T_H activity in AMLR [435c]. Deficiency in suppressor T cell function has been shown to predispose certain individuals to develop SLE, and during exacerbation of disease activity, further depression of inducible T-suppressor activity has been reported. Because of the defective suppressor activity in SLE, such patients were used as "positive controls" in suppressor assays carried out in this project. As in RA, suppressor activity normalizes in inactive disease. Like RA, SLE is a heterogeneous disease, including subtypes with different disease courses and prognoses.

CHAPTER 1.2 AIMS OF THE PROJECT

In the light of previous work in our laboratory, showing the importance of cell surface SH groups on subsequent cell function, we wished to investigate what further aspects of immunoregulation are mediated by cell surface SH groups in normal healthy controls, and then to try and relate this to defective immunoregulation as seen in RA NSAID, which appears to be improved by sulphhydrate drug treatment.

The present study was motivated by four rationales: 1] the undisputed recognition in animal and human systems that immune responses are controlled by a complex interaction of immunoregulatory cells, and their Ag- and non-Ag-specific soluble factors capable of either suppressing or helping both humoral and cell mediated immune responses [438,439]; 2] the knowledge that disturbances in number, function, or both in such cells can be associated with immunological diseases, and that autoimmune diseases characterised by immunological hyperactivity usually at the level of the B cells, are frequently associated with decreased suppressor cell activity [440]; 3] the increased oxidative metabolism observed in RA [100,100b]; 4] the decreased serum and membrane SH levels in RA [409].

Despite experiments showing the inhibitory action of H_2O_2 on the CON-A induced suppressor assay [441], the greater sensitivity of T8

cells compared to T4, monocytes, neutrophils, erythrocytes and B cells to the injurious effects of H_2O_2 [77], the plethora of papers showing reduced SH levels resulting in a defective SH/S-S redox state in RA, normalized by sulphhydrate drugs [404], and the known sensitivity of SH groups to ROS [414]. No one to date has directly looked at the relationship between all these events, and whether raised ROS leading to reduced membrane SH levels could lead to defective suppressor activity. This was the purpose of this project, hence the title "immunoregulation mediated by cell-surface sulphydryl groups". Thus the following basic questions were posed:

1] is there evidence for defective SH levels in RA?

2] is there defective suppressor activity in the same RA patients?

3] could 2] be the result of oxidation of important surface SH groups on immunoregulatory cells?

4] if defective suppressor activity is found, can it be improved in vitro by 2-ME or D-PEN, or in vivo by sulphhydrate drugs?

5] what other immunoregulatory mechanisms are SH dependent?

6] are any of 5] defective in RA? And if so, are they correctable by sulphhydrate treatment or 2-ME?

CHAPTER 2 - MATERIALS AND EQUIPMENT

2.1 MATERIALS

SUPPLIER

All tissue culture reagents, except where otherwise stated (a batch of FCS of low mitogenicity was reserved for us).

Gibco Bio-Cult Ltd.
Paisley,
Scotland.

Pokeweed mitogen [lyophilised crude preparation from Phyllolacca americana].

All chemicals except where otherwise stated (including antisera and reagents for Elisa technique. Note that BSA must be globulin and essentially fatty acid free).

Sigma Chemical Co.,
Poole,
Dorset,
England.

Ficoll-paque (density 1.077 ± 0.001 g/ml).

Pharmacia (GB) Ltd.,
Milton Keynes,

Percoll (density 1.130 ± 0.005 g/ml).

MK9 3HD,

Concanavalin-A [lyophilised from Jack bean Concanavalia ensiformis]

England.

Phosphate buffered Saline tablets
[Dulbecco "A"]

Oxoid Ltd., Wade Rd.,
Basingstoke, Hampshire,
RG 24 OPW, England.

Glucose	Boots the chemist, Bath, England.
Sodium Carbonate	British Drug Houses
Sodium Hydrogen Carbonate	Limited,
Disodium Hydrogen Phosphate	Poole, Dorset,
Potassium Dihydrogen Phosphate	England.
EDTA	
Betafluor Scintillation fluid	National Diagnostics, 45, Long Plough, Aston Clinton, Bucks., HP22 5HD, England.
Phytohemagglutinin - P (50mg/5ml) [purified from <u>phaseolus supp.</u> - lyophilised].	Difco Labs., P.O. Box 14B Central Lane, East Mosley, Surrey, KT8 0SE, England.
Mabs T3, T4, T8, T11, HNK-1, WR16, WR17, WR18, WR19, M2 (for specificity see Table 3.1).	Wessex Regional Immunology Service, Mab Unit, Southampton., England.

Mouse Mab to human interleukin-2
receptor [DAKO-IL-2R Mab]
Code M731
(for specificity see Table 3.1)

Dako Ltd., 22,
The Arcade,
The Octagon,
High Wycombe,
Bucks., HP11 2HT,
England.

Heparin Sodium (mucous) "monopurin"
1000 u/ml (preservative free).

Weddel Pharmaceuticals
Limited,
Wrexham, England.

(Methyl-³H) Thymidine code TRA 120.
Specific activity 5 Ci/mmol
Conc. 1 mCi/ml.

Amersham International
PLC, Amersham Labs.,
White Lion Rd.,
Amersham Bucks,
HP7 9LL, England.

I¹²⁵ r-Interleukin-2
code NEX-229
Specific activity 38 µCi/µg.
Conc. 33.3 µCi/ml.

New England Nuclear
Research Products,
54a Albany St.,
Boston, MA 02118, USA.

"Tago" affinity purified antihuman IgM
alkaline phosphatase conjugate.

Tissue Culture
Services, Slough,
Bucks., England.

Human r-Interleukin-2

50 μ g lyophilised h r-IL 2

Specific activity 10^7 IU/mg

HuT 102/B2 cells.

Royal free Mab to T8 (RF T8).

Rabbit Complement for ABC and DR Typing.

Giemsa Stain, May Grunwald Stain,

Depex Neutral Mounting medium

Dr. M.P. Weir,

Glaxo Group Research

Limited, Greenford Rd.,

Greenford, Middx.,

UB6 OHE, England.

Immunology Dept.

of above company.

Dr. George Janossy,

Royal Free Hospital,

Immunology Dept.,

London, England.

North East Biomedical

Labs. Ltd.,

P.O. Box 45,

Denham, Bucks.,

UB9 5PA, England.

Ho_pkins and Williams

Freshwater Ltd.,

Chadwell Heath,

Essex, England.

2.2 EQUIPMENTSUPPLIER

All tissue culture Nunc disposable plastics (U-shaped, □-shaped 96 well plates and □-shaped 24 well plates).

γ-irradiated polycarbonate U-shaped and polystyrene/polyethylene U-shaped centrifuge tubes.

Plastic γ-irradiated syringes and needles.

Nonsterile rigid polystyrene Nunc Immuno-I Elisa plates.

Scintillation vials and caps. 145 mm plugged disposable glass Pasteur pipettes.

75 mm x 1 mm plain glass capillary tubes.

Sterile Flow Cabinets.

Culture Incubator.

Gibco Biocult Ltd.,
Paisley,
Scotland.

Richardsons of
Leicester,
England.

M.D.H. Ltd.,
Walworth Rd., Andover,
Hampshire, SP10 5AA,
England.

Heraeus, England.

LP3 tubes.

Luckham Labs.
Supplies Ltd.,
Labro-Works,
Victoria Gardens,
Burgess Hill,
West Sussex,
England.

MSE Chilspin 2 centrifuge with swing out
buckets and plate spinner.

MSE Scientific
Instruments,
Sussex,
England.

MSE Mistral 2L centrifuge with swing out
buckets.

Bio-freeze tubes.

Northumbria
Biologicals, England.

Wilovert inverting microscope.

Microcare Services,
61, Blaisdon 1 Yate,
Bristol BS17 4TY,
England.

Dynatech Automatic MicroElisa Reader
MK 580.

Dynatech Instruments
California,
USA.

Dynatech Plate Shaker.

Howe pH-meter.

Howe,
Surrey, England.

Skatron Cell Harvester and filter paper.

Adhesive transparent microplate covers.

Flow Labs. Ltd.,

Irvine, Scotland.

Laborlux 12 microscope with/without facilities for fluorescent work (other microscopes used described in methods section).

E. Leitz Instruments
Limited,
Luton,
England.

Packard Tri Carb Scintillation Counter 3255.

United Technologies,
Packard, Berkshire,
England.

Gamma-Counter.

Single use millititer 5v

96 well filtration pack and vacuum holder.

Facilities used at
Glaxo Research Labs.,
Immunology Dept.,
Greenford, Middx.,
England.

7 ml sterile Bijoux bottles.

30 ml sterile blood collection bottles.

Sterilin,
43-45 Broad St.
Beddington, Middx.,
TW11 8Q2, England.

12 well multispot PTFE-coated slides.

C.A. Hendley (Essex)
Limited,
Oakwood Hill Industrial
Estate, Loughton,
Essex, England.

Milli-RO + Milli-Q water system for
double deionized water.

Millipore UK Ltd.,
Millipore House,
11-15 Peterborough Rd.,
Harrow, Middx.,
HA1 2YH,
England.

Millex non pyrogenic sterile single use
Dynagard filter units with low protein
binding - Durapore 0.22 μm porosite
membranes SL GV 025 BS.

Haemocytometer counting chamber, plain
glass slides and cover slips.

Arnold Howell Ltd.,
73, Maygrove Rd.,
West Hamstead, London,
NW6 2BP, England.

Nescofilm.

Fisons PLC Scientific
Equipment Division,
Bishop Meadow Rd.,
Loughborough,
Leics., LE11 0RG,
England.

Shandon cytospin

Southern Products Ltd.,
England.

CHAPTER 3 - METHODS

PREPARATION OF REAGENTS FOR PBMNC CELL SEPARATION AND TISSUE CULTURE

Phosphate buffered saline plus glucose (PBSG)

(for washing of cells and for incubations with reagents such as 2-ME, PHMPSA, D-PEN, etc.)

PBS tablets 10
 Glucose 1.6g
 Deionized water to 1 Litre

Then the above constituents were filter sterilized using Millipore filter units 0.22 μ m pore size into a sterile container and stored at 4 °C for up to two weeks.

Cell culture medium (RPMI 1640) plus 10% ECS

	Volume (ml)
RPMI 1640 (10 x strength)	100
7.5% Sodium Carbonate solution	27
Penicillin (5000 u/ml) plus	
Streptomycin solution (5000 μ g/ml)	20
200 mM Glutamine	20
Foetal calf serum	100
pH adjusted to 7.3 using 3M NaOH then	
Deionized water to.....	1 Litre

The above constituents were filter sterilized using Millipore filter units 0.22 μ m pore size into a sterile container and stored at 4 °C for up to two weeks.

PREPARATION OF REAGENTS FOR IgG AND IgM ELISA-ASSAYS

Coating Buffer

Sodium Carbonate 1.59 g
 Sodium Hydrogen Carbonate 2.93 g
 Sodium Azide 0.2 g
 pH adjusted to 9.6 using 3M NaOH then
 Deionized water to 1 Litre

The above constituents were stored at 4 °C for up to two weeks.

PBS plus 0.5% BSA (freshly prepared)

Bovine serum albumin 0.5 g
 PBS (x 1) to 100 ml

PBS/Tween

Tween 20 (Polyoxyethylene sorbitan
 monolaurate) 0.5 ml
 Sodium Azide 0.2 g
 PBS (x 1) to 1 Litre

The above constituents were stored at 4 °C for up to two weeks.

Diethanolamine

Diethanolamine 96 ml
 Deionized water 800 ml
 Sodium Azide 0.2 g
 Magnesium Chloride hexahydrate 0.1 g
 ph adjusted to 9.8 with 1M HCl
 Deionized water to 1 Litre

The above constituents were stored in an amber bottle for not more than two weeks at 4 °C.

Alkaline phosphatase conjugated to goat antihuman IgG (γ-chain specific) [Sigma]

Usually diluted 1:1000 in PBS/Tween but more dilute if the batch is enzymically very active.

Alkaline phosphatase conjugated to goat antihuman IgM (μ-chain specific) [Tago]

Usually diluted 1:3000 in PBS/Tween but more dilute if the batch is enzymically very active.

Goat antihuman polyvalent immunoglobulins (Antisera)

A bottle was reconstituted with 2 ml of sterile deionized water and stored at 4 °C if to be frequently used but otherwise 100 ml aliquots were placed in biofreeze vials and frozen at -20 °C until needed. When required the polyvalent Ig's were diluted 1:200 in coating buffer.

Substrate

1 pill (5 mg) of paranitrophenylphosphate per every 5 ml of diethanolamine to make a stock solution of 1 mg/ml prepared fresh just before use.

IgG and IgM standards for ELISA assays

A normal serum pool (negative for RhF activity) was used as a source of IgG and IgM standards containing 9 g/l and 1.5 g/l respectively as determined by nephelometry (by Mr. K. Case, Immunology Dept. RUH, Bath). The serum pool was aliquoted and stored at -90°C until required. For construction of ELISA standards curves a sample was thawed and diluted to 4000 ng/ml such that in each case the top standard would be 2000 ng/ml.

Purified IgG₁ (to test specificity of IgG and IgM assays)

IgG₁ was prepared by affinity chromatography by passing diluted human myeloma serum down a protein A column [1-3]. The IgG₁ bound to the protein A through the Fc region in a pH dependent fashion and was eluted by lowering the pH of the column. Serum obtained from an IgG₁ myeloma patient was centrifuged at 2000 g to remove particulate matter and then diluted to 50% with PBS (x 1). Aliquots of 4 ml of serum were then added to a protein A column in a stepwise fashion. The column was then washed with 10/150 phosphate buffer to remove unwanted contaminants. Acetic acid (1M) was then added to the top of the

column, and bound IgG₁ eluted into a small beker. The preparation was then left to dialyse in PBS (x 1) at 4 °C (with frequent changes of buffer) until the pH was neutral. The protein concentration of the sample was determined spectrophotometrically and the sample concentrated as required and stored at -20 °C. Before use an aliquot was thawed and diluted to 4000 ng/ml to test specificity in the IgG and IgM ELISA assays.

WHITE CELL COUNTING FLUID

Acetic acid 3 ml
 Methylene blue 0.1 ml
 Deionized water to 100 ml

(The acid lyses any red cell thus leaving only white cells to be counted; the dye aids cell counting)

REAGENTS FOR CELL VIABILITY MEASUREMENT

Acridine orange (AO) 0.1 mg
 Ethidium bromide (EB) 0.1 mg
 PBS (x 1) to 100 ml

Samples were aliquoted into bijoux bottles and frozen at -20 °C. After thawing an aliquot, it could be stored, wrapped in silver foil, at 4 °C.

PREPARATION OF REAGENTS FOR SERUM THIOL MEASUREMENT

Buffer A 0.1 M disodium hydrogen phosphate prepared in deionized water in an amber bottle at 4 °C.

Buffer B 0.1 M potassium dihydrogen phosphate prepared in deionized water and stored in an amber bottle at 4 °C.

0.1M Phosphate 150 ml Buffer A mixed with 25 ml Buffer B and pH adjusted to 7.4 and stored in an amber bottle at 4 °C.

Buffer C

DTNB solution a 0.8 mg/ml solution was made in buffer C and placed in an amber bottle just before use.

EDTA/Buffer C (for preparation of glutathione standard curve)

EDTA 0.01 g

Buffer C to 100 ml

Glutathione standard

Using reduced glutathione MW 307.33, 0.0307 g were dissolved in 1000 ml of EDTA/Buffer C and placed in an amber bottle, to give a stock solution of 1 mM. Dilutions were made from this in EDTA/Buffer C to give a standard curve ranging from 0.1 to 1 mM.

BUFFER FOR MAY GRUNWALD/GIEMSA STAINING

0.1M Citric Acid (21.01 g/l)

0.2M Disodium Hydrogen Phosphate (28.39 g/l)

85 ml Citric Acid plus 115 ml Disodium Hydrogen Phosphate adjusted to pH 5.75 and made up to 1 Liter with deionized water.

PREPARATION OF 2-MERCAPTOETHANOL

MW 78.1 Density 1.114 g/l Molarity 14.3M

First a 1M solution was made by diluting 0.5 ml of 2-ME with 6.6 ml of deionized water, termed solution A. Then 1 ml of solution A taken plus 9 ml of deionized water, termed solution B (0.1M). Then 1 ml of solution B plus 99 ml of deionized water, termed solution C (1 mM) - this was the stock solution stored at 4 °C.

Small aliquots of the stock solution were filter sterilized before use. Cells were treated with a final concentration of 2-ME of 50 μ M unless otherwise stated.

PREPARATION OF REAGENTS FOR FLUORESCENCE ASSAY

PBSA

PBSG containing 0.2% bovine serum albumin and 0.1% azide.

PBSG + FCS

PBSG containing 2% heat inactivated FCS + 0.1% azide.

Goat anti-mouse IgG FITC conjugate

The FITC conjugate (fluorescein-isothiocyanate isomer 1) fluoresces green. Aliquots were stored at 4 °C and just before use an aliquot was diluted appropriately or filtered through a 0.22 µm cellulose acetate filter to remove any aggregates which would otherwise bind to Fc receptors giving false positive results. By using a F(ab)₂ fragment Fc receptor binding was excluded.

Glycerol/PBS/DABCO

2.5 g DABCO was added to a solution containing 90 ml glycerol and 10 ml PBS (x 1).

PREPARATION OF REAGENTS FOR r-IL-2 FORMULATION

0.1M acetic acid

6 ml 1M acetic acid + 94 ml deionised water

0.2M BSA/phosphate buffer pH 4

0.42 g acetic acid were dissolved in 100 ml deionised water. 0.716 g disodium-hydrogen phosphate were dissolved in 100 ml deionised water. The buffer was produced by mixing 50 ml of each solution. 50 mg BSA was added to 10 ml of the buffer to make the final solution.

Reconstitution of recombinant IL-2

50 μg of lyophilised human r-IL-2 was supplied by Glaxo, of specific activity 10^7 Units/mg; thus 50 μg represented 5×10^5 Units of activity. It was suggested that it was stored at 4°C and not frozen. The 50 μg was dissolved in 10 ml 0.1M acetic acid to give 5×10^4 U/ml called "concentrated stock solution". 0.1 ml of this solution was taken and dissolved in 9.9 ml of 5 mg/ml bovine serum albumin/phosphate buffer pH 4 to give a final concentration of 5×10^2 U/ml and filter-sterilised using Millex GV 0.22 μm porosity membranes (mixed acetate membrane filters must not be used). This was called "stock solution". The additional protein was added to prevent losses of r-IL-2 which is very hydrophobic and easily sticks to vessels. 200 μl aliquots of this were stored at -70°C . Just prior to use an aliquot was thawed and diluted 1:5 in RPM + 10% FCS to give 100 U/ml, such that addition of 10 μl to 100 μl of cells in the "educated cells" assay would give a top standard of 10 U/ml (in some cases 100 U/ml was needed, then dilutions were made directly from the "concentrated stock solution").

Although r-IL-2 is a very stable protein, it stores better at acid pH and at isotonic strength, since at physiological pH and ionic strength it denatures; but it must not be diluted directly from an acid pH to a neutral pH since loss of activity will occur.

PREPARATION OF H_2O_2 SOLUTION

A range of H_2O_2 concentrations were freshly made up in PBSG just before use, and filter sterilised such that a range of desired concentrations would be achieved on subsequent dilutions in the culture vessels.

PREPARATION OF α -METHYL-MANNOSIDE (MW 194.2)

A 0.6 M solution in RPMI and 10% FCS was made up and filter sterilised and kept at 4 °C to be used within 2 weeks. A final concentration of 0.3 M seen by CON-A treated or UNT treated cells produced after the 48 hour preculture in the CON-A induced suppressor assay was achieved by adding 1 ml of cell suspension to 1 ml of this solution.

PREPARATION OF MITOGENS

CON-A, PHA, PWM, anti-CD3 Mab (without azide) were made up as concentrated stock solutions in PBSG and frozen at -20 °C until needed. Appropriate dilutions were made in culture medium just before use, such that desired concentrations would be achieved on addition to the cells in culture.

DETAILED METHODOLOGY

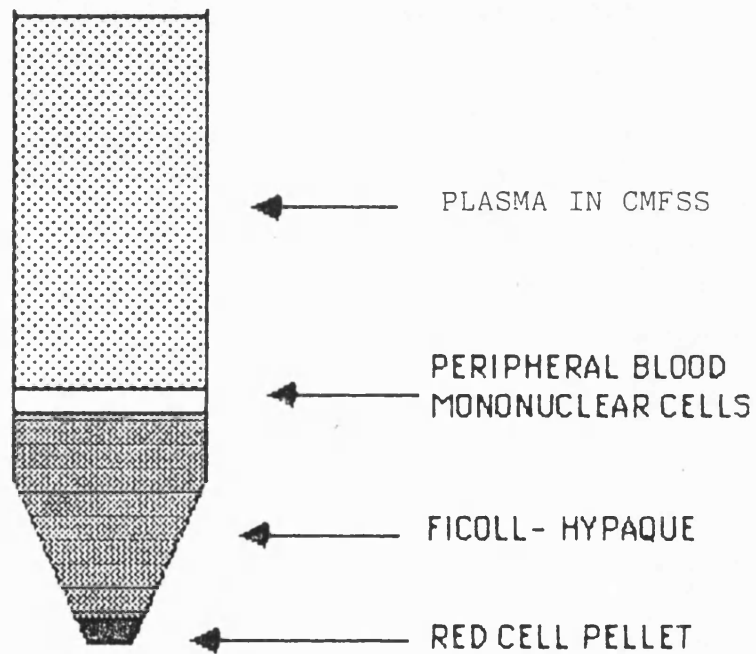
SEPARATION OF PBMNC

PBMNC were prepared from heparinised blood using a simple and rapid centrifugation technique of density centrifugation on Ficoll-Paque according to Boyum (4-8).

Blood was diluted 1:1 with PBSG (since WBC was in all cases less than $20 \times 10^9/l$) and 7 ml carefully layered onto 3 ml (pre-warmed to 20°C) Ficoll-Paque in sterile conical polystyrene-polyethylene centrifuge tubes, and centrifuged in a pre-warmed centrifuge at 18°C at 400 g for 30 min. for optimal separation (Fig. 3.1). Prior dilution of the blood improves cell yield since it reduces the size of red cell aggregates which could otherwise bring down PBMNC too. The viable PBMNC (lymphocytes, monocytes and platelets) were carefully removed from the interface using sterile disposable glass Pasteur pipettes, and placed in round bottom sterile polycarbonate tubes (to improve cell yield by inhibiting cell adherence). The aggregated red cell, granulocyte, dead cells and cell debris pellet was discarded. It is important to remove all the interface, but with a minimum of Ficoll-Paque and supernatant, since removing excess Ficoll-Paque causes granulocyte contamination and removing excess supernatant results in unnecessary contamination by plasma proteins. The PBMNC were washed 3 times with PBSG, pelleting in-between washes by centrifuging at 250 g for 10 min. to remove Ficoll-Paque and plasma, and collecting the pellet. Finally the cells were resuspended in PBSG at $5 \times 10^6/ml$ if PHMPA or 2-ME treatment were to be carried out; or

FIG. 3.1

SEPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS
ON FICOLL-HYPAQUE DENSITY GRADIENT



at an appropriate dilution in RPMI + 10% FCS if to be cultured immediately. Separation of PBMNC in this way gives cell yields of greater than 95% lymphocytes and monocytes (60% recovery of lymphocytes in original sample) with only 3% granulocytes and 5% red cells contamination, and 95% cell viability.

All separation and tissue culture procedures were performed in a flow cabinet using sterile equipment and aseptic techniques.

CELL COUNTING USING A HAEMOCYTOMETER

The cells, whether PBMNC or neutrophils were suspended in 1 ml medium or PBSG, and 20 μ l removed and mixed thoroughly with 380 μ l counting fluid in an LP3 tube.

A cover slip was stuck to the haemocytometer, and a sample of cell suspension was allowed to flow under the cover slip from a fine capillary tube until the grid area was just full but not overflowing.

The number of cells in the 2 diagonally opposed large squares were counted under 10 x magnification, and the results divided by 10 to give the number of cells $\times 10^6$ /ml.

The cells were then further diluted as necessary.

METHOD FOR CELL VIABILITY MEASUREMENT

100 μ l PBMNC cell suspension (1×10^6 /ml) was mixed with 100 μ l AO/EB solution. Cells were counted using a fluorescence microscope utilising both UV and visible illumination at the same time since the fluorescence was very strong. Acridine orange is taken up by live cells and fluoresces green/yellow. Ethidium bromide is taken up by dead cells and fluoresces orange (Fig. 3.2).

At least 200 cells were counted for accuracy.

Results were expressed as:

$$\% \text{ viable cells} = \frac{\text{Cells fluorescing green/yellow} \times 100}{\text{Total number of cells in field of view}}$$

Method based on Parks et al. [9].

LYMPHOCYTE PROLIFERATION ASSAY

100 μ l of 1×10^6 /ml PBMNC in RPMI + 10% FCS were placed in triplicate in U-shaped sterile 96-well culture plates, to which 10 μ l of mitogens appropriately diluted in RPMI + 10% FCS were added to give final dilutions of CON-A (1-100 μ g/ml) and PHA (0.01 - 1.0% of final volume, to give a range of concentrations 1 μ g/ml to 100 μ g/ml) and PWM (1:20 - 1:2000 final dilution in well), and anti-CD3 Mab (1:2000 - 1:200000 final dilution in well). To calculate background proliferation in the absence of mitogens 10 μ l of RPMI + 10% FCS was added instead. Depending on the assay, cultures were incubated for 24,

48, 96, 120, 144, 240 hours. Four hours before termination $1 \mu\text{Ci}$ of $[^3\text{H}]$ -thymidine diluted in RPMI + 10% FCS was added in a 10 μL volume. The cells were harvested onto glass fiber filter disks and oven-dried. The disks were then stored and placed into individual scintillation

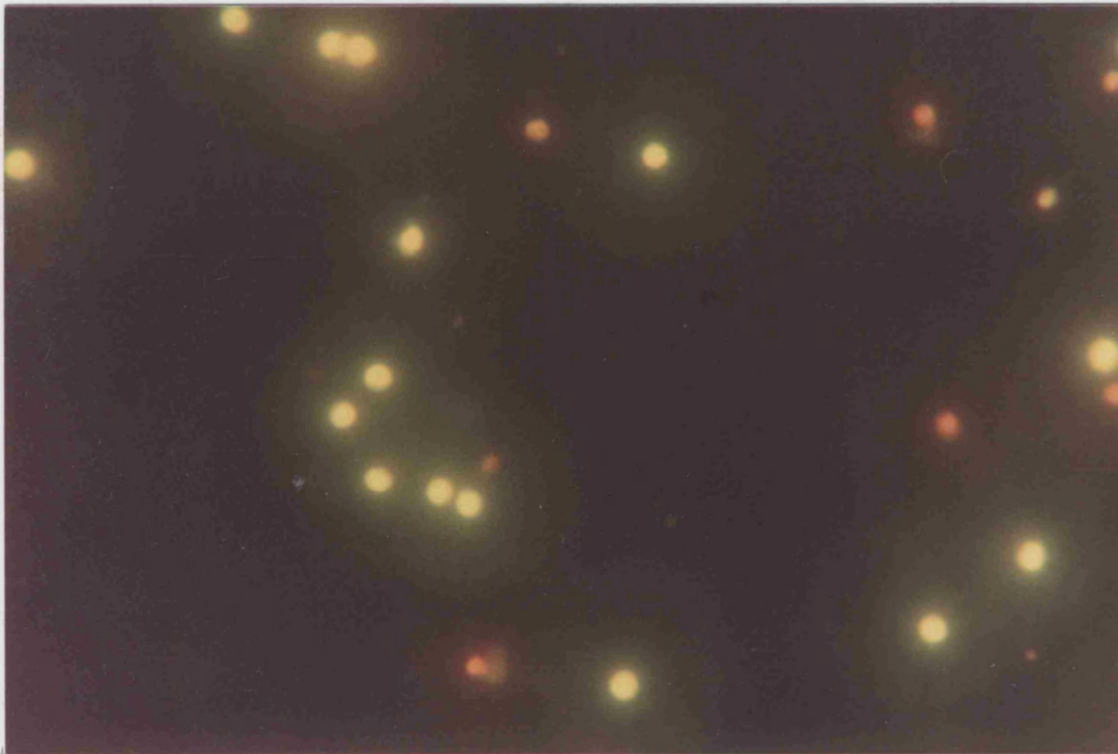


Fig. 3.2 : PBMNC CELLS STAINED WITH ACRIDINE ORANGE/ETHIDIUM BROMIDE

LIVE CELLS STAIN YELLOW/GREEN

DEAD CELLS STAIN ORANGE

MAGNIFICATION X 496

48, 96, 120, 144, 240 hours. Four hours before termination 1 μCi of [^3H]-thymidine diluted in RPMI + 10% FCS was added in a 10 μl volume. The cells were harvested onto glass fiber filter disks and oven-dried. The disks were then removed and placed into individual scintillation vials containing 2 ml of scintillant. The uptake of radioisotope was determined by liquid scintillation spectrometry. Results were considered reliable when variations in CPM was less than 10% of the mean for each triplet (to eliminate the interference due to technical errors or contamination). Finally, CPM were multiplied up so as to be expressed as CPM/ 10^6 PBMNC.

Dose-response curves over several individuals were expressed as mean of % of maximal counts for each dose.

MICROPLATE INDIRECT IMMUNOFLUORESCENCE ASSAY

U-shaped 96-well microplates were used as vehicles for simultaneously staining and washing samples [10].

Titertek multipipette and automatic micropipettes were used to allow rapidity in handling small reagent volumes and washing and resuspending of cells. Fifty μl aliquots of freshly prepared PBMNC at $2 - 4 \times 10^6/\text{ml}$ in PBSA ($1 - 2 \times 10^5$ per well) were placed in each microplate well. The plate was placed on ice for 10 min., and 5 μl of neat or appropriately diluted Mab (at 4 °C) were added per well and mixed thoroughly with the cell suspension (an optimal concentration was used giving minimal non specific binding but effective specific staining). The whole plate was covered with an adhesive transparent

cover and gently shaken on a plate shaker for 30 min. at 4 °C. The wells were then topped up with 100 µl PBSA and spun for 30 min. at 1500 RPM, then the supernatant was removed by forcefully inverting the plate onto paper towels. The cells were resuspended and washed 4 times with ice-cold PBSA. Cells were incubated for 30 min. at 4 °C with 50 µl F(ab)₂ fragment of goat anti-mouse IgG FITC conjugate (diluted 1:20) and then washed 4 times with ice-cold PBSA. After the last wash, 6 µl of PBSA was added to each of the tiny cell pellets, and 2 µl of resuspended cells transferred onto a PTFE coated multispot slide. The slide was placed in formalin vapour for 15 min., then air-dried and each well covered with a drop of glycerol/PBS (x 1)/DABCO to prevent loss of fluorescence staining [11], a cover slip was added and sealed with clear nail varnish. An excessive amount of glycerol fluid must be prevented to avoid a "halo" effect around the cells when viewed under phase; this can be prevented by applying gentle pressure to the cover slip which flattens the cells and prevents their movement. It was possible to store the slides for some months at 4 °C by keeping them in a dark box without loss of staining intensity until the slides were read. Washings and dilutions of Mab or FITC conjugate were carried out in PBSG containing 2% heat inactivated FCS and 0.1% azide to minimise non-specific Fc receptor mediated binding whenever the monoclonal was of IgG_{2a} specificity.

Control samples in the absence of monoclonal antibodies were always put up to check that there was no binding of goat anti-mouse IgG FITC to Fc receptors. Fluorescing cells were counted using a microscope with epi-illumination, filter sets for FITC, and a phase

contrast condenser. A minimum of 200 cells were counted for accurate measurement, ignoring areas of cell clumping.

Results were expressed as:

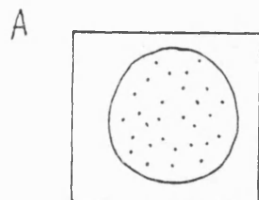
$$\% \text{ positive cells} = \frac{\text{cells fluorescing with a Mab} \times 100}{\text{total number of cells in field of view}}$$

Monoclonal antibodies form complexes with membrane marker determinants, their distribution on a cell membrane is influenced by several factors [12-13] described below. In order to prevent extensive movement of membrane components which could lead to "cap" formation (cross-linking of membrane antigens) and subsequent shedding of "caps" or endocytosis of membrane receptors, 0.2% sodium azide (a metabolic inhibitor) was added to reagents, and the cells were kept on ice to prevent shedding or endocytosis which are energy-dependent processes.

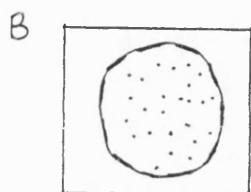
Nevertheless minimal membrane movement (patch formation) does occur even in the presence of azide; in fact it is beneficial since it helps to identify genuine membrane staining, but is distinct from the binding of IgG aggregates or complexes to cells that express strong Fc receptors, which in the latter case appear as little "lumps" that seem to be lifted out from the plane of the membrane and do not tend to show a linear apposition.

Also, rarely, viable cells can show a weak but perfect ring staining which is an artifact, sometimes due to overconjugated commercial reagents. Dead cells stain homogeneously and very brightly (Fig. 3.3).

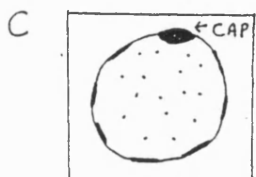
FIG3.3 TYPES OF FLUORESCENCE OBSERVED



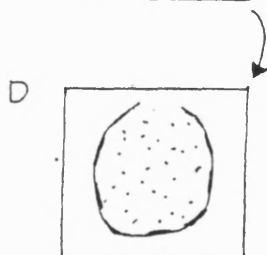
Ag IS EVENLY DISTRIBUTED OVER THE CELL SURFACE,
WHEN CELLS ARE PRE-FIXED IN FORMALIN



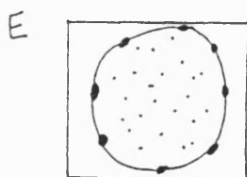
CROSS LINKING OF Ag's BY Mab IN THE PRESENCE OF
AZIDE AT 4°C CAUSES PATCH FORMATION



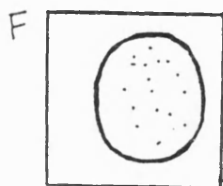
WITHOUT THE PRESENCE OF AZIDE AND $> 4^{\circ}\text{C}$
PATCH FORMATION CAN BECOME 'CAP FORMATION'...



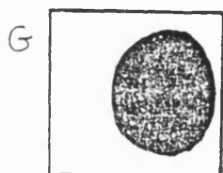
... WHICH IN TIME IS EITHER
SHED OR ENDOCYTOSED



SOLUBLE Ig AGGREGATES BIND TO Fc RECEPTOR
AND CAUSE NON SPECIFIC FLUORESCENCE



PERFECT RING STAINING INDICATES
OVERCONJUGATED FITC



DEAD CELLS STAIN VERY BRIGHTLY ALL OVER

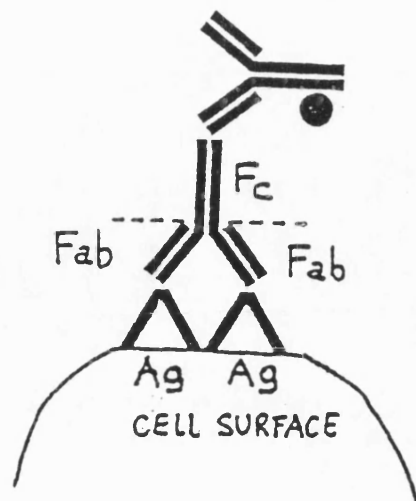
THE AIM OF GOOD IMMUNOFLUORESCENT LABELLING IS TO OBSERVE CELLS OF B MORPHOLOGY.

Indirect immunofluorescence (sandwich method) was used [where the first layer antibody was non fluorescing unmodified Ig, and the second layer antibody directed against the first was fluorescing] since it is a more sensitive assay (Fig. 3.4). It is important to wash the samples well when removing excess Mab since residual traces of the primary antibody in the supernatant can form soluble complexes with the second antibody and bind to Fc receptors on irrelevant cells.

It has been suggested that to avoid variations due to different batches of reagents and monoclonals [14] and day to day observer variations, PBMNC can be stored frozen at -70°C , in 10% DMSO and in liquid nitrogen and successfully thawed without loss of T-cell markers [15-16].

However it was decided not to do this, but to prepare slides immediately, store them as described, and read them when convenient. Table 3.1 shows all the Mab's used throughout this project, including their Ig subclasses and specificity.

FIGURE 3.4



Indirect Method.

Key

 *Tissue Antigen*

 *Primary Antibody*

 *Secondary Antibody*

 *Visual Marker*

DIAGRAM REPRESENTING THE INDIRECT IMMUNOFLUORESCENT TECHNIQUE.

TABLE 3.1 MONOCLONAL ANTIBODIES USED

REFERENCES

Mab name	Commercial or other source equivalent	CD no.	Ag MW Kda	Specificity	% Positive PBMC
Soton T3 Subclass IgG2a	UCHL1 Leu-4 OKT3	CD3	19	T-cell receptor on mature T-cells >95% PB T-cells	70-80
Soton T4 Subclass IgG2b	OKT4 Leu-3 Hetero- antisera TH ₂	CD4	60 62	H/I T-cell subset for T-T, T-B, T-Mac etc. interactions 65% PB T-cells	40-60
Soton T8 Subclass IgG2a	OKT8 Leu-2 Hetero- antisera TH ₂	CD8	31-33 when reduced with 2-ME 76-78 when oxidized	S/C T-cell subset 35% PB T-cells	20-30
Soton WR16 (1) Subclass IgG1	2H4	CD45R	200	S/I or "naive" subset of T4 that suppress B L ϕ Ig production (only 50% T4 ⁺ , but also 71% B and 80% T8 ⁺ . On 86% WR19 ⁻ CD4 ⁺ . Restricted to L ϕ .	>40-60 Strong staining
Soton WR17 (2) Subclass IgG2		CD37	40 45	Pan B L ϕ ⁻	\cong 20
Soton WR18 (3)	---	unas- signed		HLA class II β chain DP, DQ and DR on B L ϕ , M ϕ , macrophages, subset of null cells and activated T-cells	20
Soton WR19 (1) Subclass IgG1	4B4	CDW29	135 (?)	H/I or "memory" subset of T4 i.e. 43% T4 ⁺ , not to T8 or B L ϕ . On 89% WR16 ⁻ CD4 ⁺ . Also expressed on cells of non-lymphoid lineage	\cong 20-30 low intensity staining
Soton T11 Subclass IgG2a	9.6 Leu-5 OKT11	CD2	45-50	Sheep E rosette +ve T-cells, i.e. pan-T cell	\cong 83
Soton M2 Subclass IgG2b	Leu-15 OKM1 Mac-1 anti-Mol	CD11 (C3b Rec)	170	M ϕ , granulocytes, Null cells (NK), T-suppressor and large granular L ϕ (LGL) mainly myelomonocytic cells	\cong 25
Soton NHK-1	Leu-7	CD 57	110	LGL, NK and K cells and some T8 +ve cells	20

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24 - 25

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TABLE 3.1 (continued)

Soton Mabs supplied as mouse ascites fluid preserved with sodium azide. None of the Soton Mabs are able to fix C'. WR17 can fix rabbit, not human C'. 10% T4 express neither WR16 nor 19. Wessex region Mabs WR16 and 17 were secreted from hybridomas derived by fusion of the NS-Omyeloma cell line and splenocytes from BALB/c mice hyperimmunized with chronic lymphocytic leukaemia cells. WR19 Mab was secreted from a hybridoma derived by fusion of the NS-O myeloma cell line and splenocytes from mice hyperimmunized with the human T-cell line HuT 78. OK series from Orthodiagnosics Ltd. Leu series from Becton Dickinson. UCHT-1 from University College.....others from Coulter Diagnostics Ltd.

REFERENCES

Monoclonal antibody	CD no.	Ag MW Kda	Specificity
DAKOPATTS IL-2R Mab Supplied as tissue culture supernatant containing sodium azide Clone ACT-1 79 µg/ml (IgG1, Kappa conc.)	CD25	55 (Tac Ag)	Reacts with IL-2R (without inhibiting its function), expressed on mitogen/ bacterial/viral activated T-cells and B-cells (at a lower density). T + NK cells activated in MLR. Staining pattern said to be essentially identical to that of anti-TAC.

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INDUCTION OF IL-2 RECEPTOR

PBMNC were diluted to 1×10^6 /ml in RPMI + 10% FCS and cultured in 1 ml aliquots for 72 hours with/without either 10 μ g/ml Con-A or 0.1% PHA v:v (optimal final concentrations), in U-shaped 24-well culture plates, and harvested with a rubber policeman, washed and diluted to appropriate concentrations.

EFFECT OF PHMPA ON ABILITY OF r-IL-2 TO INHIBIT ANTI-TAC BINDING AS ASSESSED BY FLUORESCENCE

Cells were harvested after IL-2 receptor induction, and diluted to 5×10^6 /ml in PBSG and treated with/without 50 μ M PHMPA for 1 hour at 37 °C, followed by thorough washing and dilution to 4×10^6 /ml in PBSG.

50 μ l samples were added to U-shaped wells in 96-well microplates, and 5 μ l of r-IL-2 added to give a final concentration of 1 μ g/ml and incubated for 1 hour at 37 °C with the cells.

Excess r-IL-2 was then washed off, and cells diluted to 4×10^6 /ml in PBSA and treated with 5 μ l IL-2 receptor Mab (supplied as 79 μ g/ml) neat or diluted 1:10 or 1:1000, and then the cells were treated as instructed in Immunofluorescence method stated previously..

In all cases the following wells were set up:

MITOGEN TREATED CELLS FOLLOWED BY NO PHMPA

	+ Mab (neat/diluted)	- Mab
	+ FITC	+ FITC
cells treated with no r-IL-2	should show max. fluorescence	control for non specific binding by FITC - should show no fluor.
cells treated with r-IL-2	should show reduced or no fluorescence	"

MITOGEN TREATED CELLS FOLLOWED BY PHMPA

	+ Mab (neat/diluted)	- Mab
	+ FITC	+ FITC
cells treated with no r-IL-2	to show effect of PHMPA on CD25 expression	control for non specific binding by FITC - should show no fluor.
cells treated with r-IL-2	to verify PHMPA inhibition of r-IL-2 binding and therefore loss of r-IL-2 inhibition of Mab binding	"

Two wells were set aside to measure background fluorescence in non mitogen stimulated cultures treated with/without PHMPA, and these values were subtracted from appropriate wells containing mitogen stimulated cells, such that fluorescence solely due to stimulation with mitogen could be calculated.

PRODUCTION OF r-IL-2 CONTAINING SUPERNATANTS

PBMNC (1×10^6 /ml) were incubated as 1 ml cultures in RPMI + 10% FCS in biofreeze tubes with loosened tops, with/without Con-A (10 μ g/ml, shown to be optimal) in a humidified atmosphere of 5% CO₂ in air at 37 °C for 24 hours. At the end of the incubation period, the cells were washed twice in RPMI + 10% FCS containing 0.3M α -methyl-mannoside (to get rid of bound Con-A) and once in RPMI and 10% FCS and then the cells were resuspended in 1 ml of medium and incubated for a further 24 hours without mitogen. IL-2 output peaked at 48 hours, thus a final total culture period of 48 hours was used. The tubes were then centrifuged at 2000 RPM for 15 min., and supernatants collected and frozen at -20 °C prior to assay.

CYTOSPIN PREPARATION FOR LOOKING AT CULTURES AFTER CON-A INDUCED SUPPRESSOR CELL GENERATION

Suppressor cells were generated in the usual 48-hour Con-A generating system, and then harvested using a rubber policeman. Cells were concentrated to 1×10^6 /ml and 4 drops of untreated or Con-A treated effector cells suspension were added to separate funnels in the cytospin machine. The cytospin was set to spin at 400 - 450 RPM for 10 min., and in this time a jet of cells was shot onto named glass slides; excess moisture was removed by filter paper attached to the slide. The cells were fixed in formalin vapour and air-dried and then stained using May-Grunwald/Giemsa stain.

Fixed cells were immersed in buffer for 2 - 5 min., then transferred to May-Grunwald stain (diluted freshly 1:2 with buffer) for 5 min. The slides were then rinsed by flushing buffer over them, and finally the cells were stained in Giemsa solution (freshly diluted 1:5 in buffer) for 15 min., and excess stain removed with buffer.

To make a permanent preparation for photographic purposes, the cells were mounted in neutral DEPEX mounting medium under a cover slip, which was sealed with nail varnish.

A SIMPLE MICROASSAY TO GENERATE "EDUCATED CELLS" FOR QUANTIFICATION OF IL-2 ACTIVITY IN MITOGEN-STIMULATED CELL SUPERNATANTS

Based on a method by Istvan Ando [29].

A simple short term culture in vitro assay was used to quantitate human r-IL-2 based on spontaneous "educating" of peripheral blood T-cells in tissue culture by FCS and subsequent expression of the IL-2 receptor after 10 days. The results obtained with this assay agree with those of assays which utilise IL-2 dependent T-cell clones (e.g. CTLL-2), but its advantage is that it is highly sensitive, (unlike RIA [30-31] or ELISA [32] methods, which are 10 to 50 times less sensitive than bioassays) down to pg concentrations (physiological concentrations) thus suitable for this study where low concentrations were expected. Other advantages are that it is reproducible, easy to perform, less prone to mycoplasma contamination

(it is difficult to find mycoplasma-free cell line stocks, and such contamination reduces sensitivity, accuracy, and reproducibility of IL-2 assays [33]) and does not need to be grown continuously - as cytotoxic T-lymphoid lines (e.g. CTLL-2) do, since they lose viability on freezing and thawing -, nor does it require the cost of maintaining mice if a mouse CON-A blast system was to be used.

PBMNC were cultured in 2 ml volumes in RPMI + 10% FCS in U-shaped 24-well plates for 10 days at 0.5×10^6 /ml in the absence of mitogens. Cells were harvested with a rubber policeman and termed "educated cells" since they now bear high affinity IL-2 receptors.

Use of "educated cells" to quantify IL-2 in supernatants

The "educated cells" were harvested using a rubber policeman, and washed 3 times in RPMI + 10% FCS, then diluted to 0.2×10^6 /ml. 100 μ l were added to appropriate wells in U-shaped 96-well plates. The cells were cultured for 4 days in the presence or absence of 10 μ l r-IL-2 in various final doses 1000 - 0.001 U/ml to give a standard curve to which IL-2 in sample supernatants could be compared. The cells were pulsed with 1 μ Ci/well [3 H]-thymidine ([3 H]-TdR) for the last 4 hours of the culture period, and the amount of [3 H]-TdR incorporated was measured using a β -scintillation counter.

When assaying sample supernatant, 10 μ l RPMI was added to wells 2 to 6. 10 μ l neat supernatant was added to well 1 and 10 μ l supernatant double-diluted in RPMI from wells 2 to 6, giving final dilutions of 1:10 to 1:320, since 100 μ l "educated cells" was added to each well.

IL-2 in supernatants was calculated from individual titration curves using a point on the curve causing maximal stimulation of "educated cells", or any point on the curve that double-diluted out and results expressed as IU IL-2/ 10^6 PBMNC.

"Educated cells" have the advantage that they can be frozen in medium + 10% DMSO without loss of activity, so long as they are only thawed once; thus, all supernatants can be studied in one batch, if so desired.

THE EFFECT OF PHMPA ON 125 I-IL-2 BINDING TO IL-2R ON HuT 102/B2 CELLS (which express IL-2R at high density)

PHMPA was solubilised in PBS (x 1) and incubated for 1 hour at 37 °C with 4×10^6 cells/ml at a final concentration of 50 μ M. The cells were then washed 3 times in RPMI + 10% FCS + 0.5% azide, and then diluted to 4×10^6 cells/ml and 50 μ l (2×10^5 cells) were aliquoted to wells in the filtration plate to which either 50 μ l of medium or cold IL-2 (1 μ g/ml) was added, and left for 20 min. at room temperature (found to be equally good as 1 hour at 37 °C), followed by 50 μ l of 125 I-IL-2 (5 nCi/well) for 45 min. at room temperature (found to be equally good as 30 min at 37 °C). After 3 washes in RPMI + 10% FCS + 0.5% azide to remove unbound label, using the 96-well microtiter filtration plate (having a porosity of 5 μ m permitting removal of unbound label by applying vacuum to the underside of the plate).

The plate was then oven-dried at 60 °C for 30 min.. Filter disks

from each well were then punched out and counted by a γ -counter. All samples were in duplicate or quadruplicate, and CPM expressed as the average of these.

The cold IL-2 served as a positive control to test for specificity, and the medium served as a negative control. Specificity of ^{125}I -IL-2 interaction with the IL-2 receptor was shown by finding that cold transferrin or cold IL-1 do not inhibit ^{125}I -IL-2 binding, whereas cold IL-2 does. For this, bound ^{125}I -IL-2 was visualised directly onto an autoradiograph by sandwiching an X-Ray film between the plate and an intensifying screen, left in a sealed black plastic bag at -70°C for 48 hours. The X-Ray film was developed after 2 days, and inhibition of ^{125}I -IL-2 binding to HuT cells was indicated by an area of less exposure on the autoradiograph.

The effect of PHMPSA on subsequent binding of hot IL-2 was expressed as relative % proliferation defined as:

$$\left[\frac{\text{CPM after PHMPSA treatment} \pm \text{rIL-2}}{\text{CPM before PHMPSA treatment} \pm \text{rIL-2}} - 1 \right] \times 100$$

where a negative % represents inhibition of hot binding and a positive % represents enhanced hot binding, 0% represents no effect by PHMPSA

RED CELL LYSING SOLUTION FOR PBMNC

(to prepare PBMNC for certain CON-A induced suppressor assay experiments and for fluorescent studies wherever necessary)

Stock (x 10)

Ammonium chloride..... 8.260 g

Potassium bicarbonate..... 1.000 g

Tetrasodium EDTA..... 0.037 g

Deionised water..... to..... 50 ml

Adjust pH to 7.3

Deionised water..... to..... 100 ml

Working solution (x 1)

Dilute stock 1:10 with deionised water.

Both solutions stored at room temperature in tightly stoppered containers.

Discard both after 1 week.

2 ml lysing solution was added to the PBMNC pellet and vortexed immediately and left to incubate at room temperature (or if necessary at 37 °C) for 5 to 10 min. until lysis was complete and the supernatant appeared transparent red. This procedure was repeated if there was still red cell contamination; and the lysing solution removed by washes in PBSG followed by centrifugation at 400 g for 5 min.

COMPLEMENT MEDIATED CELL CYTOLYSIS USING Mab RF T8

Certain Mab's can bind complement and lyse cells which bear the Ag for which the Mab is specific. This methodology was used to lyse CD8⁺ cells produced in the Con-A stimulated suppressor cell generating system of the Con-A induced suppressor assay.

Con-A and untreated effector cells were counted and concentrated into pellets which were resuspended in neat RFT8 by adding $30 \mu\text{l}/10^6$ cells, these were then left at 4 °C for 30 min. (shown by viability and fluorescence marker studies to be a ratio which is 100% efficient in removing T8 cells, but not to affect viability). During this period, rabbit complement was allowed to warm to room temperature, and then reconstituted in 1 ml deionised water, and filter sterilised into sterile bijoux bottles.

An equal volume of rabbit complement was added to cells + RF T8 (class IgM). For a complement control, samples of untreated or Con-A treated cells were treated with complement alone, replacing the Rf T8 volume with an equal volume of RPMI + 10% FCS. The cells were left with complement for 45 - 60 min. at 37 °C, and then washed 3 times in medium and used in the usual way in the Con-A induced suppressor assay.

Until use, RF T8 and rabbit complement must be stored frozen at -20 °C, thawed once, and then discarded.

SERUM THIOL LEVELS MEASURED USING ELLMAN'S REAGENT (DTNB)

[34].

3-5 ml venous blood was withdrawn and placed into a plain glass tube and allowed to coagulate at room temperature for 1 hour before centrifuging to separate serum. Serum samples were stored at -20°C for 2 months before use. Stored samples do show a fall in serum thiols compared to fresh samples, but this fall plateaus out by 2 months, thus all samples are comparable again at this point [35].

A 10 μl sample was reacted with 170 μl of 0.1 M phosphate buffer C pH 7.4, and 20 μl of freshly prepared 0.8 mg/ml 5,5'-dithio-bis(2-nitrobenzoic acid) [DTNB].

The reaction vessel was U-shaped wells in a 96 microwell NUNC Immuno T plate.

Sample blanks were prepared by addition of 10 μl sample to 190 μl buffer C. A positive low control serum, in the form of newborn calf serum, was used in each run to check for inter-batch variability. The wells were covered with Nescofilm, and incubated at 37°C for 20 min..

The absorbance of the yellow thionitrobenzoic acid (TNB-SH) was read at 440 nm on a Dynatech ELISA reader, at pH 7.4 this constitutes total serum thiol levels i.e. both "slow" and "fast" reacting SH groups. 1 mole TN-SH is liberated by reaction of 1 mole serum SH with DTNB.

Serum thiol levels were calculated by reference to a standard curve (0.1 - 1 mM; Fig. 3.5). The standard curve was set up rapidly because glutathione is quickly oxidised in the air, and would give falsely low results. However, once the reaction is completed the colour intensity is very stable as seen in Fig. 3.6.

POKEWEED MITOGEN (PWM) DOSE-RESPONSE CURVE

PWM (a polyclonal non specific B-cell activator) was used to stimulate PBMNC to produce immunoglobulins as supplied as a freeze-dried sample which was reconstituted with 4 ml sterile deionised water. 200 μ l aliquots of this stock solution were frozen at -20°C in sterile bijoux bottles until use.

Prior to an experiment an aliquot was thawed and diluted appropriately, such that addition of 10 μ l to 200 μ l cultures of cells at 1 or $2 \times 10^6/\text{ml}$ would give final dilutions ranging from 1:20 to 1:2000. In samples where no PWM was added, an equivalent volume of RPMI + 10% FCS was included.

Cultures were carried out in U-shaped 96-well culture plates.

The cultures were placed in a 37°C humidified incubator for usually 1 week, being constantly gased with a mixture of air/5% CO_2 , and supernatants harvested and frozen at -20°C until IgG/IgM production was measured.

FIG. 3.5 GLUTATHIONE STANDARD CURVE FOR
MEASUREMENT OF THIOL GROUPS

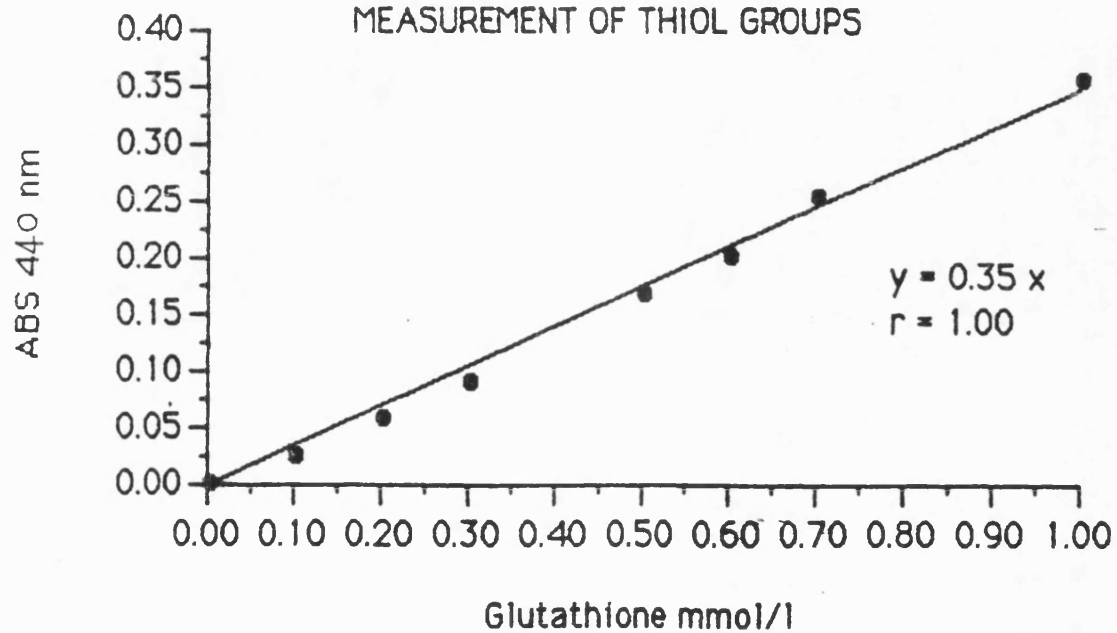
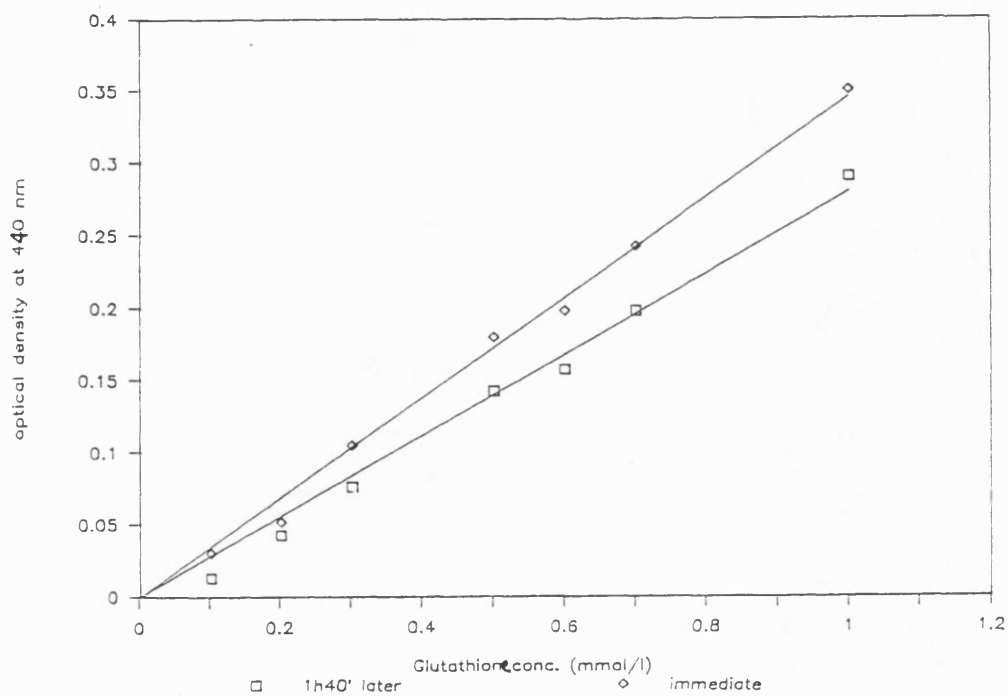


FIG. 3.6 STABILITY OF END POINT



ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Supernatants from cell cultures were harvested and assayed for the presence of IgM or IgG by ELISA.

100 μ l of an optimal dilution (1:200 in coating buffer) of goat anti-human polyvalent Ig were dispensed into each well of a 96-well NUNC Immuno I ELISA plate. The plate was covered with Nescofilm and incubated for 1 hour at 37 °C under humid conditions. The plate was either used immediately or stored frozen until needed, prior to the 1 hour incubation step.

The plate was then washed 3 times with coating buffer to get rid of excess polyvalent, and fluid forcefully removed by inverting the plate onto paper towels.

100 μ l PBS/BSA was added to each well, and the plate covered with Nescofilm to prevent edge effects. BSA prevents non-specific binding of IgG or IgM in the supernatant.

PBS/BSA was left in the wells for 1 hour at 37 °C in a humid atmosphere, then 100 μ l of each culture supernatant was double-diluted across 6 wells.

100 μ l of a standard IgG/IgM containing solution was double-diluted from positions 2 to 12 in the first row of the plate, well A1 serving as a reagent blank.

The plate was covered with Nescofilm and incubated for 1 hour at 37 °C in humid conditions, then the wells were washed thoroughly with

PBS/Tween and tapped dry onto paper towels.

100 μ l alkaline phosphatase conjugated goat anti-human IgG (diluted 1:1000 in PBS/Tween) or anti-human IgM (diluted 1:3000 in PBS/Tween) was added to each well, and the plate covered with Nescofilm and incubated for 1 hour at 37 °C in humid conditions, followed by extensive washing with PBS/Tween and thorough tapping out of excess conjugate.

100 μ l alkaline phosphatase substrate i.e. p-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer pH 9.6, was added to each well, and the colour allowed to develop at room temperature without covering.

Once the top IgG or IgM standard had reached an absorbance of 1.0 at 405 nm, the plate was rapidly read in a Dynatech micro-ELISA reader. IgG/IgM concentration of unknown supernatants were determined by reference to the standard IgG/IgM calibration curve (2 to 2000 ng/ml), simultaneously constructed.

Calculations were aided by the use of a computer program that could plot standard curves and eliminate unwanted points not on the linear portion of the curve, in order to construct the line of best fit through the remaining points. Using such a standard curve, supernatants concentrations were automatically calculated, and a printout of results produced. Results were obtained in ng/ml supernatant and since this contained 10^6 PBMNC, results were finally expressed as ng/ 10^6 PBMNC.

SHORT-LIVED SUPPRESSOR ASSAY (SLSA) [36]

PBMNC were isolated as usual, 100 μ l of 1×10^6 /ml suspension placed in U-shaped 96-well microtiter plates. Some samples were stimulated immediately with various concentrations of Con-A (0, 0.1, 1, 5, 10, μ g/ml) for 96 hours, other samples were left for 24 hours with no mitogens, and then stimulated with mitogen for a further 72 hours.

Experiments for each set of conditions were carried out in triplicate. In the last 4 hours of culture, 1 μ Ci [3 H]-TdR was added per well.

Suppressor activity was expressed as a suppressor index (SI), given by the formula:

$$SI = \frac{\text{CPM for mitogen stimulated culture including 24 h. no mitogen pre-culture period} - \text{CPM for non mitogen stimulated culture including 24 h no mitogen pre-culture period}}{\text{CPM for mitogen stimulated culture with no pre-culture period} - \text{CPM for non mitogen stimulated culture with no pre-culture period}}$$

A 24 hour pre-culture period had been shown to be optimal, since if longer culture periods were used, unstimulated cell counts became very high and SI dropped due to subtraction of these high values.

CONCANAVALIN-A INDUCED SUPPRESSION IN A DOUBLE CULTURE
SYSTEM (Fig 3.7)

The lectin Concanavalin-A in addition to its ability to induce lymphocyte transformation [37] and "lectin dependent" lymphocyte cytotoxicity [38], also stimulates a lymphocyte subpopulation to generate suppressor cell activity [39]. Two indicator systems were used to demonstrate this inducible suppression:

- A] A PWM B-cell stimulated system to investigate Con-A induced suppression of IgG or IgM synthesis
- B] A PHA stimulated blast cell system to investigate suppression of T-cell proliferation by Con-A induced suppressor cells.

CON-A induced suppressor activity as measured by suppression of IgG or IgM synthesis [40,40b].

PBMNC were separated and diluted 1×10^6 /ml in RPMI + 10% FCS and stimulated with/without 10 μ g/ml Con-A. After 48 hours the cells were harvested with a rubber policeman and washed once with culture medium and 3 times with medium containing 0.3M α -methyl-mannoside (to remove cell bound CON-A), and once again in α -methylmannoside free medium.

Con-A treated cells were termed "Con-A effector cells" and non-mitogen treated cells termed "UNT - effector cells". Both populations of effector cells were adjusted to 2×10^6 /ml.

CONCAVALIN-A INDUCED SUPPRESSOR CELL ACTIVITY

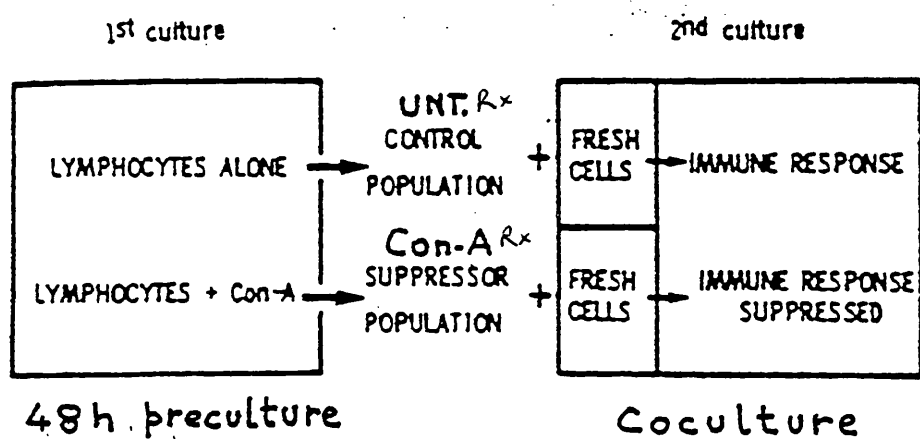


FIGURE 3.7 Diagrammatic representation of the double culture assay for concanavalin-A induced suppression.

100 μ l of each were separately mixed with 100 μ l of freshly prepared autologous PBMNC at 2×10^6 /ml, to give an effector:responder coculture ratio of 1:1.

Cells were cultured in U-shaped 96-well microtiter plates in the presence of PWM 1:200 dilution for 1 week. Supernatants were harvested and IgG and IgM contents assessed by ELISA (Fig. 3.8).

IgG and IgM synthesis in the CON-A induced suppressor assay was expressed as relative % Ig synthesis as defined by the following formula (this method was felt to visually depict suppression more closely)

$$\text{relative \% Ig synthesis} = \frac{\left[\begin{array}{l} \text{Ig (ng/10}^6\text{Resp.) from Con-A coculture stimulated with 1:200 PWM} \\ \text{Ig (ng/10}^6\text{Resp.) from Con-A coculture in absence of PWM} \end{array} \right] - 1}{\left[\begin{array}{l} \text{Ig (ng/10}^6\text{Resp.) from UNT - coculture stimulated with 1:200 PWM} \\ \text{Ig (ng/10}^6\text{Resp.) from UNT - coculture in absence of PWM} \end{array} \right]} \times 100$$

Ig was expressed as ng/ 10^6 responder cells.

Therefore a negative result indicated % suppression of IgG or IgM synthesis, and more precisely -100% represented complete suppression of Ig synthesis relative to that in the UNT coculture; a positive result indicated % enhancement of IgG or IgM synthesis relative to that in the UNT coculture; zero % indicated that IgG or IgM synthesis

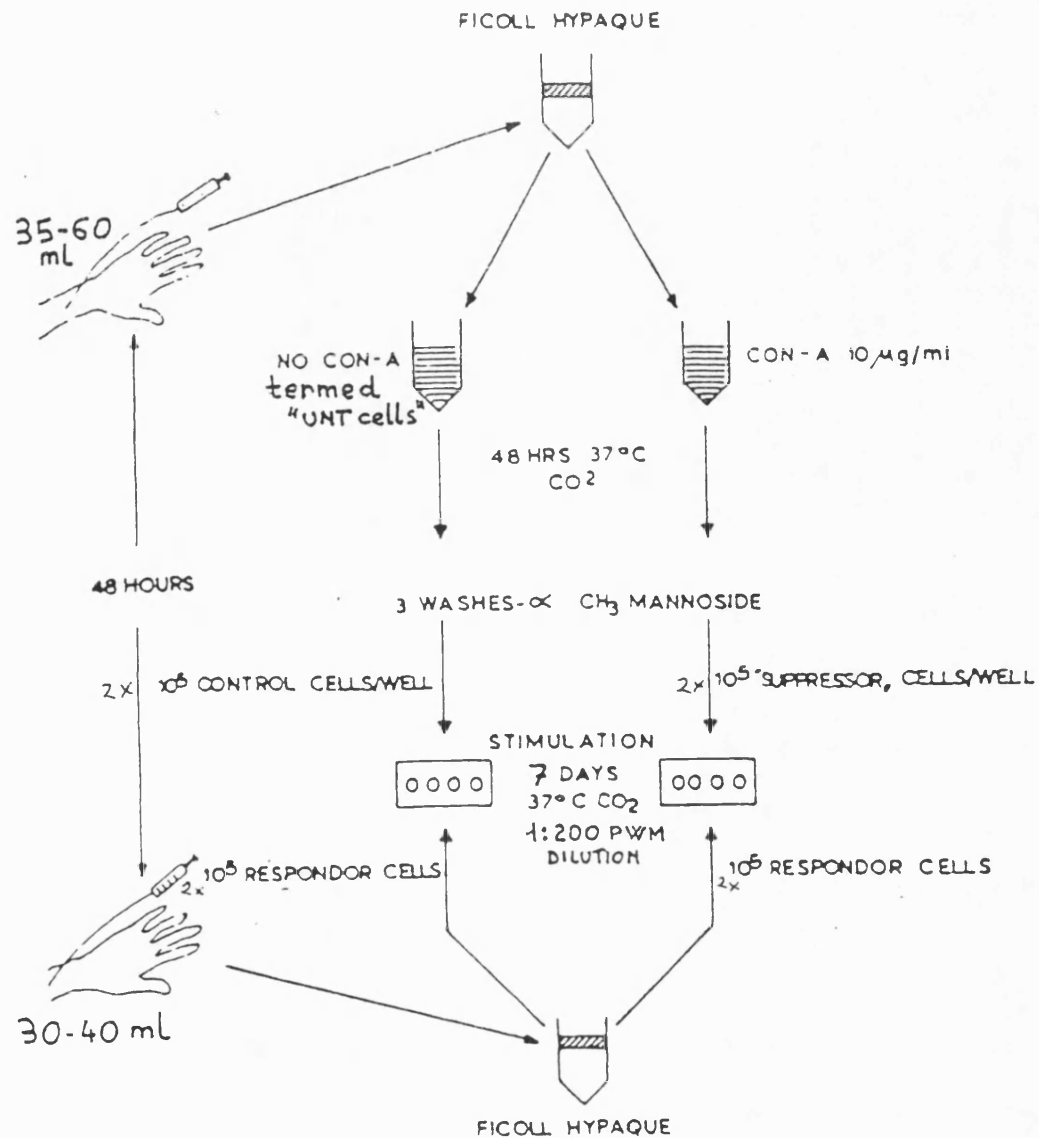


FIGURE 3.8- Protocol followed in the double culture model for Con-A induced suppression.

Suppressor cells being demonstrated by assessing their effects on Ig synthesis from B-cells in response to PWM, a non specific polyclonal B-cell activator.

in the CON-A coculture was equivalent to that in the UNT coculture.

CON-A induced suppressor activity as measured by suppression of proliferation [39]

Con-A induced putative suppressor effector cells were generated as usual- Both UNT and Con-A effector cells were treated with Mitomycin C (50 $\mu\text{g/ml}$) for 35 min. at 37 °C, and then washed 3 times with 0.3M α -methyl-mannoside in RPMI + FCS (to remove cell bound Con-A) which would otherwise stimulate responder cells in the coculture. Cells were washed once more in culture medium and resuspended at $1 \times 10^6/\text{ml}$.

100 μl of both effector cells suspension were separately mixed with 100 μl of freshly prepared autologus responder cells (at $1 \times 10^6/\text{ml}$) to give an effector:responder ratio of 1:1.

Culture were set up in triplicate, and stimulated with 0.1% PHA (v/v), shown to be an optimal mitogenic dose for proliferation, and cultured for 72 hours.

The cells were pulsed with 1 μCi [^3H]-TdR 4 hours before the end of the culture period, and [^3H]-TdR incorporation was detected by β -scintillation counting (Fig. 3.9).

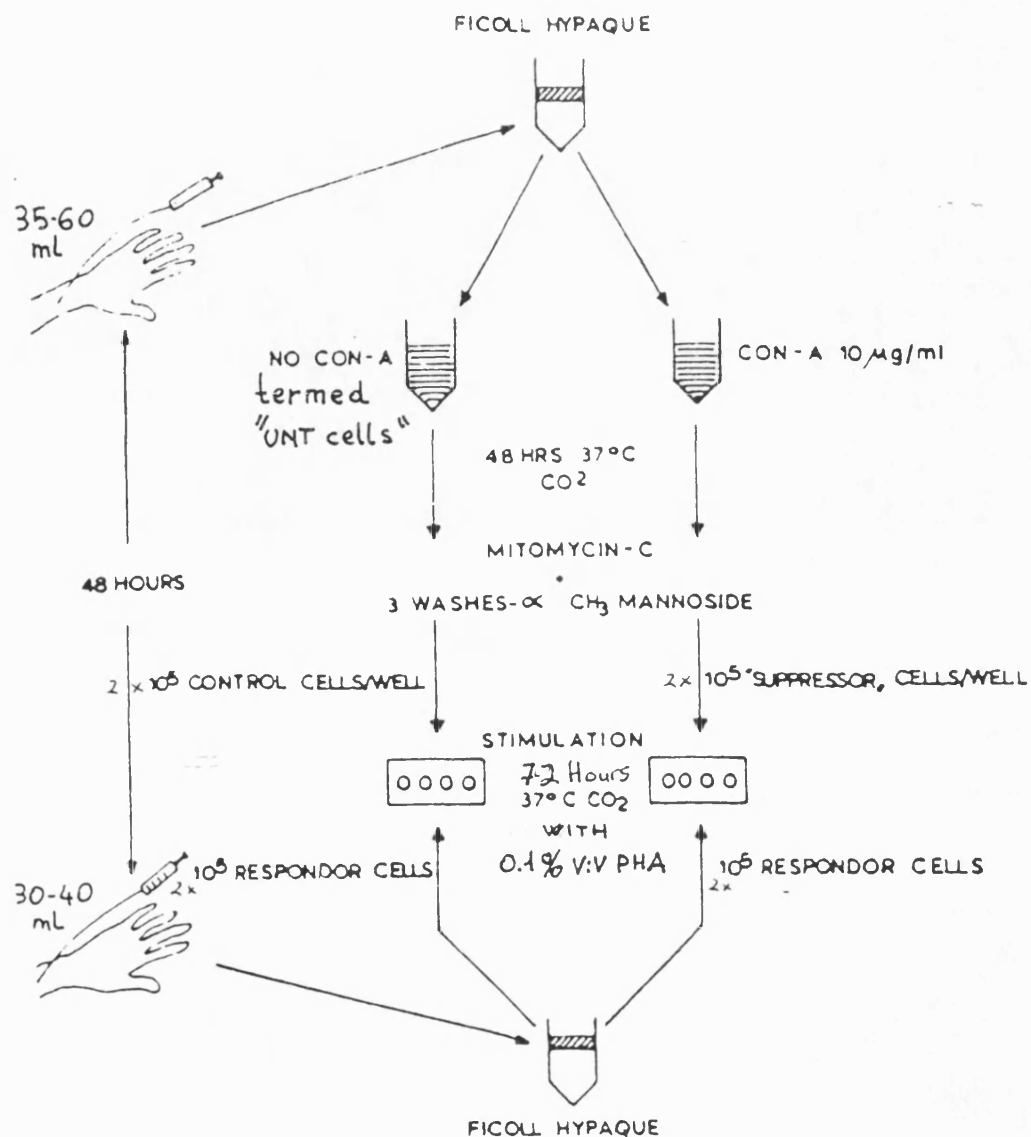


FIGURE-3.9 Protocol followed in the double culture model for Con-A induced suppression.

Suppressor cells being demonstrated by assessing their effects on lymphocyte proliferation in response to the non specific mitogen PHA.

Cell proliferation in the CON-A induced suppressor assay was expressed as relative % proliferation as defined by the following formula:

$$\text{relative \% prolif.} = \frac{\left[\begin{array}{cc} \text{CPM of Con-A} & \text{CPM of Con-A} \\ \text{coculture in} & \text{coculture in} \\ \text{presence of PHA} & \text{absence of PHA} \end{array} - \frac{\left[\begin{array}{cc} \text{CPM of UNT} & \text{CPM of UNT} \\ \text{coculture in} & \text{coculture in} \\ \text{presence of PHA} & \text{absence of PHA} \end{array} \right]}{\text{CPM of UNT coculture in presence of PHA}} - 1 \right] \times 100$$

Where a negative result indicated suppression of proliferation, and more precisely - 100% represented complete suppression of proliferation relative to that in the UNT-coculture. A positive result indicated % enhancement of proliferation relative to that in the UNT-coculture, and a zero result indicated that proliferation in the CON-A coculture was equivalent to that in the UNT-coculture.

Controls were always carried out to check that mytomicin-C caused 100% inhibition of proliferation of Con-A or Untreated effector cells. Thus, the only cells capable of proliferating in coculture were fresh responder cells.

PREPARATION OF REAGENTS FOR NEUTROPHIL SEPARATION6% Dextran solution

Dextran (MW 500,000) 6 g
PBSG (x 1) to 100 ml

Stored at 4 °C

PBS (x 1 strength)

PBS tablets 10
Deionized water to 1 Liter
Filter sterilized and stored in sterile container at 4 °C for up to
two weeks.

PBS (x 10 strength)

PBS tablets 10
Deionized water to 100 ml
Filter sterilized and stored in sterile container at 4 °C
for up to two weeks.

PBSG + Ca²⁺/Mg²⁺ Buffer

PBS tablets 10
 Glucose 1.6 g
 Calcium Chloride 0.096 g
 Magnesium Chloride 0.290 g
 Adjust pH to 7.3 with 3M NaOH
 Deionized water to 1 Liter
 Filter sterilized and stored in sterile container at 4 °C for up to
 two weeks.

Percoll solution (Iso-osmotic) Ref [41-45]

Sterile PBS (x 10 strength) 10 ml
 Sterile Stock Percoll to 100 ml
 Stored in a sterile container at 4 °C for up to two weeks.

65% V/V Percoll solution (density 1.083 g/ml)

Sterile Iso-osmotic Percoll 65 ml
 Sterile PBSG (x 1) 35 ml
 Adjusted to a density of 1.083 g/ml with the use of a sterilized
 hydrometer and stored in a sterile container at 4 °C.

Red cell lysing fluid

Ammonium Chloride 0.83 g
 Deionized water to 100 ml
 Filter sterilized and stored in sterile container at 4 °C.

PREPARATION OF REAGENTS FOR SUPEROXIDE ASSAY

Superoxide Dismutase (SOD) [freshly prepared]

2.0 mg of SOD was added to 2 ml of PBSG (x 1) to give a stock solution of 3000 u/ml which when diluted 1:10 in the reaction vessel gave a final concentration of 300 u/ml.

Ferricytochrome C [freshly prepared]

To 25 ml of (PBS + $\text{Ca}^{2+}/\text{Mg}^{2+}$) 33.3 mg of ferricytochrome C were added to give a stock solution of 107 μM which when diluted 1:1.43 in the reaction vessel gave a final concentration of 75 μM .

Preparation of stimuli

FMLP and PMA were dissolved in DMSO and stored frozen at -70°C until required. Just before use aliquots were thawed and diluted in PBSG to a desired concentration. The final DMSO concentration in the reaction vessel was never allowed to exceed 0.01%; this concentration had no anti-oxidant activity against superoxide generated by a Xanthine/Xanthine oxidase system.

Cytochalasin-B

A stock solution of 0.5 mg/ml in deionized water was prepared and aliquots placed in eppendorf tubes and frozen at -20°C until needed.

Just prior to use an aliquot was thawed and 10 μl added to 1 ml of neutrophils to give a final concentration of 5 $\mu\text{g}/\text{ml}$ shown to stimulate maximal superoxide release, but not to reduce neutrophil viability [46].

NEUTROPHIL SEPARATION AND PURIFICATION

Neutrophils were purified by density gradient centrifugation of erythrocyte depleted whole blood using a modified method of Segal et al (1980) [47].

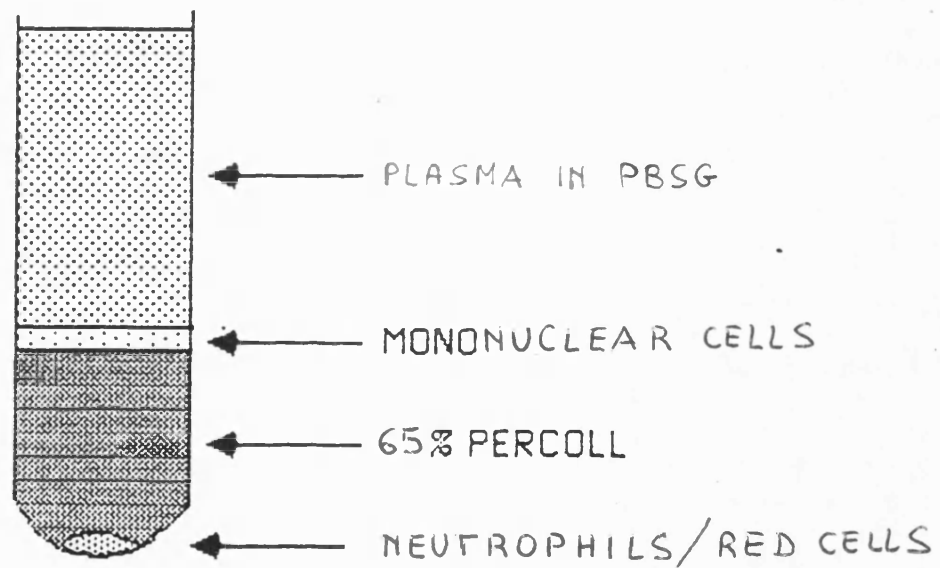
Heparinised venous blood (4 u/ml preservative free heparin) was diluted 50:50 with PBSG. 8 ml of diluted blood were then thoroughly mixed with 2 ml of 6% dextran in U bottomed polycarbonate centrifuge tubes (which unlike glass or polystyrene prevent adherence and artifactual neutrophil stimulation [48]) and allowed to stand for 30 minutes at room temperature, after which time the majority of the erythrocytes had sedimented to the bottom of the tube. The leukocyte-rich "buffy-coat" suspension was then carefully layered onto 2 ml of percoll (density 1.083 g/ml) and centrifuged for 30 minutes at 400 g at room temperature.

The separation process separated two cell populations as shown in the accompanying diagram (Fig. 3.10). Mononuclear cells remained in a band at the interface between the percoll and plasma whilst granulocytes passed through the percoll layer to form a pellet with any residual erythrocytes. The granulocytes prepared in this way were considered to contain a negligible number of eosinophils and basophils and so were therefore termed "neutrophils" from now on in the text.

The cell pellet was then carefully isolated, transferred to a new polycarbonate centrifuge tube and washed once in PBSG. After washing, the PBSG was removed and the cell pellet was resuspended in 2 ml of sterile 0.83% w/v ammonium chloride red cell lysis fluid to lyse the

FIG. 3.10

SEPARATION OF MONONUCLEAR CELLS & NEUTROPHILS
ON 65% PERCOLL



few remaining red cells. The cells were left in contact with ammonium chloride for exactly 10 minutes at 37 °C (shown not to alter neutrophil viability) and then rapidly neutralised by adding 10 ml of PBSG. After a further two washes in PBSG and centrifugation at 150 g the cells were resuspended in 1 ml of PBSG and counted by a light microscopy in white cell counting fluid and subsequently diluted to 10×10^6 /ml in PBSG.

Cells prepared in this way were consistently > 95% polymorphonuclear leukocytes as assessed by morphology (that is the easily identifiable neutrophil) and > 95% viable as assessed by acridine orange/ethidium bromide.

SUPEROXIDE ASSAY METHODOLOGY

Using a modified method of Pick et al [49]

This method is based on the reduction of ferricytochrome-C by superoxide, the product being measurable at 550 nm.

10 µl cytochalasin-B was added per ml of cell suspension to give a final concentration of 5 µg/ml. The cells were incubated for 10 minutes at 37 °C to maximize superoxide output and then added with the following agents to LP3 reaction tubes to give final concentrations per tube as follows: 75 µM ferricytochrome C, 300 u/ml SOD, 1×10^6 /ml neutrophils, $\pm 5 \times 10^{-8}$ M FMLP or ± 10 ng/ml PMA, found in preliminary experiments to be optimally stimulatory (see fig. 4.125, 4.126) and 0.01% DMSO.

To stimulated test samples without added SOD

Ai 700 μ l ferricytochrome C
 100 μ l of PMA or FMLP
 100 μ l of PBSG
 100 μ l of neutrophils

To stimulated test samples with added SOD (stimulated control)

Bi 700 μ l ferricytochrome C
 100 μ l of PMA or FMLP
 100 μ l of SOD
 100 μ l of neutrophils

To resting samples without SOD

Ci 700 μ l ferricytochrome C
 200 μ l PBSG
 100 μ l neutrophils

To resting samples with SOD (resting control)

Di 700 μ l ferricytochrome C
 100 μ l PBSG
 100 μ l SOD
 100 μ l neutrophils

Reagent blanks (Aii, Bii, Cii, Dii respectively) were prepared by replacing the 100 μ l of cells in the above with PBSG. All tubes were incubated at 37°C for 10 minutes, then placed on ice for 5 minutes and spun for 10 minutes at 150 g at 4 °C. 200 μ l of supernatants were carefully transferred to 96 F-Nunc Immuno-I plates and absorbances read on an ELISA reader at 550 nm. Final absorbances were calculated by first subtracting appropriate reagent blanks.

$$(A_i - A_{ii}) = W$$

$$(B_i - B_{ii}) = X$$

$$(C_i - C_{ii}) = Y$$

$$(D_i - D_{ii}) = Z$$

Then subtracting absorbances in the presence of SOD from those in its absence such that:

$$W - X = P \quad \text{absorbance due to superoxide released in stimulated sample}$$

$$Y - Z = Q \quad \text{absorbance due to superoxide released in unstimulated sample}$$

Finally subtracting the absorbance due to unstimulated cells from that of stimulated cells i.e. $P - Q = R$, which is the absorbance solely due to superoxide released in response to FMLP or PMA.

The presence of SOD serves solely to prove that the substance that is detected is actually superoxide. Since the concentration of SOD is sufficient to dismutate all superoxide that could possibly be produced by neutrophils under these conditions, the resultant absorbance of Bi-Bii and Di-Dii should always be zero. Absorbances can then be converted to nmol ferricytochrome C reduced over the 10 min.

incubation period using the following formula:

$$A = E \times C \times L$$

Beer-Lambert's law, where

A = Absorbance

C = Concentration (mM)

L = Length of light path (cm)

E = Extinction coefficient of ferricytochrome C

($29.5 \text{ mM}^{-1} \text{ cm}^{-1}$)

The length of the light path (L) was determined by measuring the radius (r) of the ELISA plate wells and then applying the formula:

$$\text{Volume} = \pi \times r^2 \times h$$

where h is the height of the liquid in the well and therefore equivalent to the length of the light path (L).

$$\begin{aligned} 0.2 \text{ cm}^3 &= 3.142 \times 0.349^2 \times h \\ &= 0.3848 \times h \end{aligned}$$

$$\text{therefore } h = 0.520 \text{ cm}$$

$$\begin{aligned} \text{Absorbance} &= 29.5 \times C \times 0.52 \\ &= 15.34 \times C \end{aligned}$$

$$\text{therefore } C = A / 15.34$$

giving the concentration as mM

to convert C to μM or nmol/ml $\times 10^3$.

Results were expressed as nmol/ml cytochrome-C reduced by 10^6 cells in 10 minutes.

BLOCKADE OF CELL SURFACE SULPHYDRYL GROUPS ON NEUTROPHILS OR PBMNC

Cell surface sulphydryl groups were blocked with incubation with p-hydroxy-mercuriphenylsulfonic acid (PHMPSA, an irreversible non penetrating thiol blocking agent) [50].

Neutrophils or PBMNC were diluted to 5×10^6 /ml in PBSG and half treated with 50 μ M PHMPSA (unless otherwise stated) and the remaining half left untreated. Both samples were then incubated for 1 hour at 37 °C followed by 3 washes with PBSG, the cells were pelleted in-between washes by spinning at 250 g for 10 min., and then neutrophils were resuspended in PBSG at a final concentration 10×10^6 /ml prior to the superoxide assay, but the PBMNC were resuspended in RPMI + FCS at an appropriate concentration depending on the assay for which they were to be used.

Where the effect of PHMPSA on Ig synthesis was considered, relative % Ig synthesis was defined as follows:

$$\text{relative \% Ig synthesis} = \left[\frac{\text{Ig synthesis (1:200 PWM-BKG) from PHMPSA-Rx cultures}}{\text{Ig synthesis (1:200 PWM-BKG) from untreated cultures}} - 1 \right] \times 100$$

Ig expressed as ng/ 10^6 PBMNC

Where the effect of PHMPSA on proliferation was considered, relative % proliferation was defined as follows:

$$\text{relative \% prolif.} = \left[\frac{\text{CPM (in presence of mitogen)-BKG in PHMPA-Rx cultures}}{\text{CPM (in presence of mitogen)-BKG in untreated cultures}} - 1 \right] \times 100$$

proliferation expressed as CPM/ 10^6 PBMNC

Where the effect of PHMPA on superoxide production was considered, relative % superoxide production was defined as follows:

$$\text{relative \% superoxide production} = \left[\frac{\text{O}_2^{\cdot -} \text{ production in presence of stimulant - BKG from PHMPA-Rx neutrophils}}{\text{O}_2^{\cdot -} \text{ production in presence of stimulant - BKG from untreated neutrophils}} - 1 \right] \times 100$$

$\text{O}_2^{\cdot -}$ production expressed as nmol/ml cytochrome-C

reduced by 10^6 cells in 10 min.

In all cases a negative % indicates suppression has occurred, a positive % indicates enhancement, a zero % indicates that PHMPA has no effect on the parameter under consideration.

SOURCE OF EXPERIMENTAL BLOOD (taken after informed consent)

Normal healthy controls

All experimental material has been kindly donated by either staff or students at the Bath Arthritis Research Centre or staff at the RNHRD.

Patients

AS, PSA, SLE and most RA material was taken from in-patients. The patients selected for each disease group fulfilled their respective diagnostic criteria as defined by the American Rheumatism Association (ARA). Classical and definite RA being defined in reference [51], definite AS and PSA as defined in reference [52] and definite SLE as defined in reference [53].

Some RA samples were taken from out-patients. All RA NSAID patients had active synovitis, according to the referring physician, with warm, red, painfully swollen synovial joints. He or she also assigned presence (and severity) or absence of synovitis to the other patient groups.

Great difficulty was found in recruiting patients in the RA NSAID group, since many potentially suitable patients could not be used because of recent intake of steroids, which are known to be immunosuppressive and would thus invalidate the results. Others were too anaemic for use in these studies since about 100 ml of blood were required for the suppressor assay. Since these patients were

undergoing repeated blood testing for clinical reasons, further blood subtraction for research purposes was felt not ethically acceptable, since worsening anemia could have resulted, and iron replacement therapy is known to worsen inflammation of the joints, and it has been found that the amount of lipid peroxidation products in synovial fluid correlate with the concentration of Fe salts present and with the disease activity [54].

Thus, only patients with > 10 g/dl Hb were used, which meant probably most severely ill patients were omitted. Others on NSAIDs were omitted because of systemic vasculitis associated with less active synovitis. Furthermore, it is the policy of the RNHRD to put RA patients with severe synovitis onto second line drugs as rapidly as possible to try and slow down the disease process.

Difficulties were also encountered in recruiting patients on D-PEN or Aurothiomalate alone, since very often with severe active unremitting synovitis patients are put on combined treatment D-PEN and steroids, making them unsuitable for this project.

Other patients in this group were excluded because of vasculitis or Hb < 10 g/dl.

SLE patients were used as positive controls in the CON-A induced suppressor assay.

AS/PSA patients were grouped together and used as arthritic controls.

All blood was collected by venopuncture by trained phlebotomists, quantity taken depended on the experiment for which blood was to be

used, and ease of bleeding. Usually 20 ml was taken for fluorescence studies; 30-40 ml for production of educated cells; 15-30 ml for proliferation or IL-2 production studies; and for the CON-A induced suppressor assays, 40-60 ml was taken for the 48 hour preculture system, 35-40 ml was taken at a second bleeding for responder cells used in the coculture; 3-5 ml clotted blood was taken for serum thiol estimation. 20 U heparin was added per ml blood collected. Samples were all taken between 0830 and 0930 to minimize artifacts due to circadian rhythms [55-57]. Blood was separated and analysed as quickly and efficiently as possible to retain a maximum number of healthy functional cells, since storage of whole blood for 24 hours at room temperature has been reported to result in reduced lymphocyte yield, artifactual increase in Null cells, altered expression of surface markers, and reduced response to mitogenic stimulation. These alterations probably begin soon after blood collection [58-60].

It has also been shown that variables which might influence circulating T-cells subsets - of relevance to this project - are: smoking [61], and stress (as manifested by increased circulating plasma adrenaline concentration [62]).

For all experiments RA D-PEN/AU patients have been considered together since they showed similar thiol, proliferation and CON-A induced suppressor activity.

For the same reason in all experiments AS and PSA patients have been considered together.

Patient data was collected using the following form (Table 3.2).

TABLE 3.2 PATIENT DATA COLLECTION FORM
(Boxes to be ticked where appropriate)

Permission to take blood given by doctors/nurses in charge of patient ☐

Patient gave informed verbal consent for his/her blood to be taken ☐

Name

Hospital Registration Number

D.O.B. Sex ...

Disease

Disease duration, since diagnosis (years)

PMH

Relevant FH

Pharmacological history

.....

.....

Duration and dose of present drugs

.....

.....

Clinical indices of disease activity;

Active ☐ Inactive ☐ Active peripheral synovitis ☐

EAD eg. Nodules (N), Vasculitis (V), Carpal Tunnel Syndrome (CTS), Bakers Cyst (BC), Sjögren's Syndrome (SS).

☐

Sero-ve ☐ Sero+ve ☐ RF (IU/ml)

EMS (min.)

Clinical indices of acute phase response;

ESR (mm/h)

CRP (g/l)

Viscosity (cp)

WBC ($\times 10^9/l$)

PLTS ($\times 10^9/l$)

Other parameters;

L \emptyset ($\times 10^9/l$)

Hb (g/l)

Serum -SH levels ($\mu\text{mol/l}$)

Serum IgA (g/l) IgG (g/l) IgM (g/l)

HLA status

Antinuclear Abs (ANA)

Mitochondrial Abs

Smooth muscles Abs

Reticulum Abs

Gastro. parietal Abs

Date blood taken

For experiment:

QUICK CLASSIFICATION FOR EASY REFERENCE

DISEASE	RF STATUS	BASIC RX
RA <input type="checkbox"/>	+Ve <input type="checkbox"/>	NSAID <input type="checkbox"/>
AS <input type="checkbox"/>	-Ve <input type="checkbox"/>	DPEN/AU <input type="checkbox"/>
PSA <input type="checkbox"/>		Other <input type="checkbox"/>
SLE <input type="checkbox"/>		

STATISTICAL ANALYSIS

All data was considered not normally distributed (unless otherwise stated), therefore non-parametrical statistics were applied. Paired data was analysed using Wilcoxon signed rank test. Unpaired data was analysed using the Mann-Whitney U-test, unless otherwise indicated.

Correlations between parameters were assessed by the linear regression analysis and coefficients of correlation (r) quoted. A two sided p value of < 0.05 was considered statistically significant in all analyses.

CHAPTER 4: RESULTS

4.1. COMPARISON OF SERUM THIOL LEVELS IN VARIOUS PATIENT GROUPS AND NORMALS.

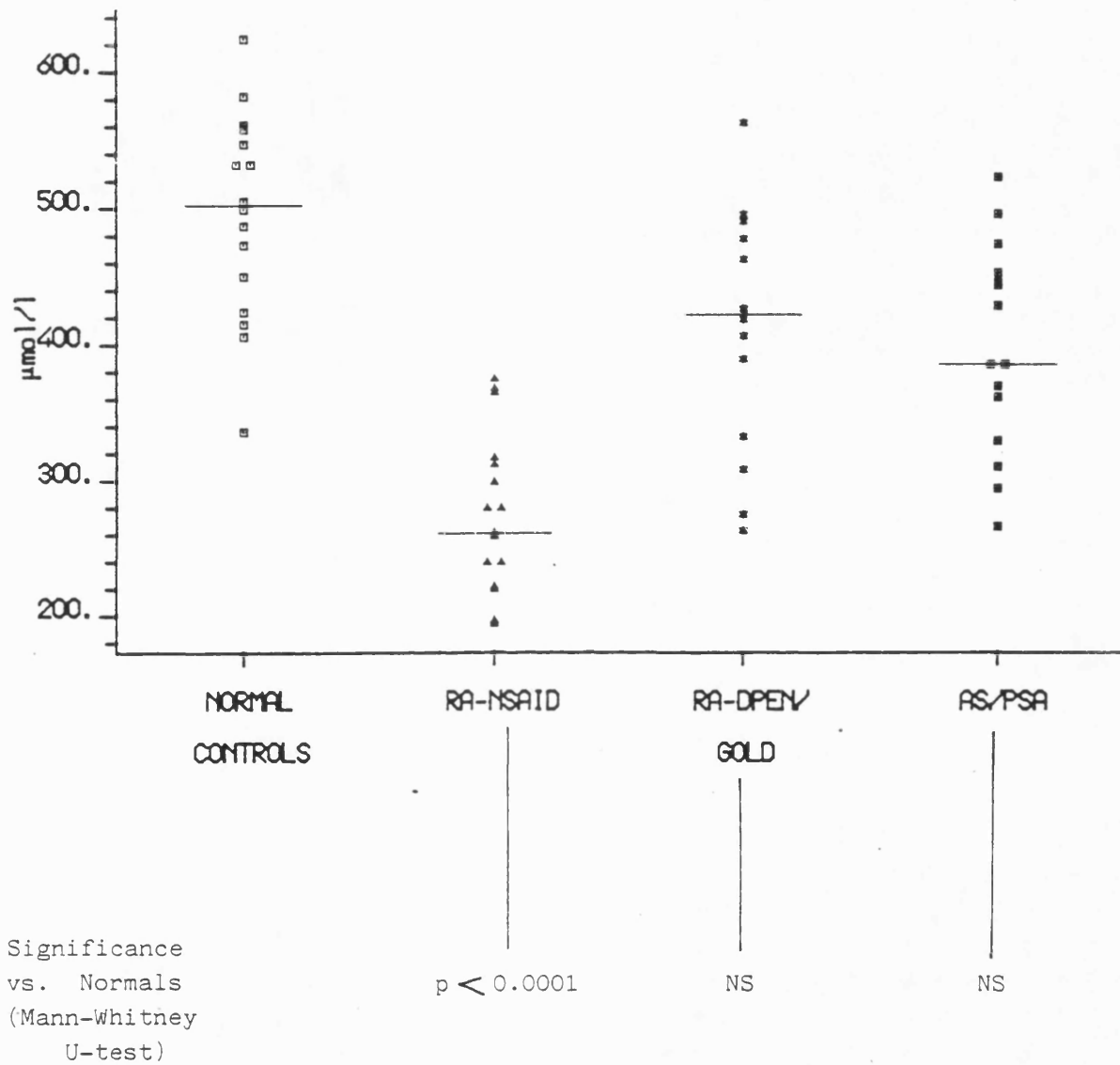
Since the aim of this project was to investigate what aspects of immunoregulation are mediated via cell surface -SH groups - and thus whether defective immunoregulation as seen in RA could be partly the consequence of oxidized functionally important -SH groups, we first sought to show reduced -SH levels in RA NSAID patients. Since one would expect reduced free -SH groups to extend from soluble proteins (mainly on albumin) to -SH groups on proteins integrated into cell membranes, as evidenced by a trend towards correlation between these two parameters shown by Mc Keown et al. [1], and since measurement of the former is simpler and requires less blood, this measurement was carried out using a modified method based on that by Ellman [2]. Since no significant correlation was observed between serum thiols level and RF, all patients +/- RF were grouped together.

It was clearly demonstrated that RA NSAID showed significantly reduced serum thiol levels, but that RA-DPEN and AS/PSA patients were not significantly different to normal controls, as shown in Fig. 4.1.

Serum thiols are known to fall with age but only significantly after the age of seventy, age is probably not the reason for these differences since the median age was less than seventy in all groups and those patients who showed most significant reduction in thiol

FIG. 4.1

SERUM THIOL LEVELS IN HEALTHY CONTROLS
AND VARIOUS PATIENT GROUPS



Horizontal bars represent median serum thiol concentration.

levels were not usually the oldest patients.

Median age and range for normals was 35 (20-70) for RA NSAID, 65 (36-82) for RA D-PEN/AU, 49 (37-78) for AS/PSA, 48 (29-79).

No significant correlation was found between serum thiols and age in normals, nor in RA NSAID, nor in RA-DPEN/AU, nor in AS/PSA; likewise no significant correlation was found between serum thiols and CRP nor WBC in RA NSAID, which often rise in acute inflammatory phases.

Although not significant, there was a trend towards a correlation between thiol levels and length of time on the sulphydrate drugs ($n = 13$, $r = 0.65$), suggesting that the longer a patient is on a sulphydrate drug, the more serum thiol levels are normalized. No serial studies in individuals were carried out to prove this, although others have found this to be true [3-5]. However, it has been shown that sulphydrate drugs normalise serum thiol levels by increasing fast reacting thiol groups, but not significantly influencing slow reactive thiol groups [6], and it is the latter that are more deficient in RA patients [6], thus this could in part account for the inability of these drugs to cure the disease.

Two basic approaches can be used to study immunoregulatory cells: one consists of "phenotypically" quantitating in tissues or PBMNC preparations, lymphocyte subsets with putative suppressor or helper properties, or alternatively looking at "functions" of immunoregulatory cells.

4.2. PHENOTYPIC COMPARISONS OF PBMNC IN RA NSAID AND NORMALS

Identification of such subsets has been attempted in this project by means of monoclonal antibodies to different surface markers on PBMNC (Table 4.1) showing no significant differences between percentages of lymphocytes, or monocytes or B-cells in RA NSAID samples compared to normals, nor between percentages of CD_3^+ , CD_4^+ , CD_8^+ , $CDW29^+$, $CD45R^+$ and a rough estimate of $CD_4^+CD45R^+$ T-cells; however, neutrophil contamination of the PBMNC preparation was more common in RA NSAID samples, probably because of prior activation in vivo resulting in reduced density, evidence for this idea of prior activation comes from the observation of raised DR and TAC expression on RA NSAID cells. There was a tendency for RA NSAID patients to show reduced CD_8^+ and more CD_4^+ cells, indicated in the raised CD_4/CD_8 ratio, suggesting that a regulatory lymphocyte imbalance may be present in some of the individuals.

Samples of synovial fluid and synovial membrane were not available for similar analyses to be carried out on them. However, due to functional heterogeneity within phenotypically defined subsets, and because phenotypic markers do not tell the experimenter anything about the functional ability of the cell in question, a more functional approach for looking at immunoregulatory T-cells was preferred, as used in this project.

Thus it was decided to investigate the thiol dependence of suppressor cell activity in normals and whether this is defective in RA NSAID utilizing various suppressor cell assays:

TABLE 4.1

CELL SUSSETS IN PBMNC PREPARATIONS OF NORMALS AND RA NSAID PATIENTS.

Mab SPECIFICITY	Mab USED	NORMALS MEDIAN (RANGE)	RA NSAID MEDIAN (RANGE)	p VALUE (M-W-U-TEST)
CD2	SOTON T11	n=5 84 (75-90)	ND	--
CD3	SOTON T3	n=9 65 (42-80)	n=7 65 (45-86)	NS
CD4	SOTON T4	n=9 55 (34-58)	n=7 53 (31-67)	NS
CD8	SOTON T8	n=9 24 (20-27)	n=7 19 (10-35)	NS
CD4:CD8	SOTON T4:T8	n=9 2.0 (1.5-3.0)	n=7 2.6 (2.0-3.5)	NS
CD45R	WR16	n=6 67 (40-80)	n=5 60 (30-79)	NS
VERY ROUGH ESTIMATE OF T_4^+ WR16 ⁺ = (WR16 ⁺ - [T8 + B1])		30 (25-40)	25 (25-50)	NS
SINCE NOT ALL T8 ⁺ STAIN WITH WR16				
CDW29	WR19	n=3 22 (22-35)	n=5 25 (23-40)	NS
CD25	ANTI-TAC DAKOPATTS	n=5 0 (0-11)	n=7 7.2 (1-20)	< 0.001
HLA CLASS II β CHAIN [DP, DQ, DR]	WR18	n=7 22 (19-31)	n=7 30 (20-35)	< 0.05
CD37 ON B-CELL	WR17	n=3 23 (11-24)	n=7 23 (12-25)	NS
MAINLY MØ	SOTON M2	n=4 24 (15-25)	n=7 24 (21-36)	NS
MAINLY NK CELLS	SOTON HNK-1	n=3 19 (4-26)	ND	--
ROUGH ESTIMATE OF NK CELLS = (T3 ⁺ - T11 ⁺)		20 (10-31)	ND	--

NEUTROPHIL CONTAMINATION

MORE COMMON HERE

- a) Concanavalin-A induced suppressor activity-suppression being assessed by measuring suppression of immunoglobulin synthesis in a double culture system.
- b) Concanavalin-A induced suppressor activity-suppression being assessed by measuring suppression of proliferation in a double culture system.
- c) Non inducible suppressor cell activity - suppression being assessed by observing the loss of short-lived suppressor cells, thought to give an indirect assessment of spontaneous suppressor cells [7].

Attention was concentrated on the assay a) rather than b), since it was decided that this best illustrates the "state" of the immune system in an individual, since for immunoglobulin synthesis to occur requires cooperation between the two arms of the immune system, that is the Humoral and Cell-mediated system and associated soluble factors, e.g. Interleukins.

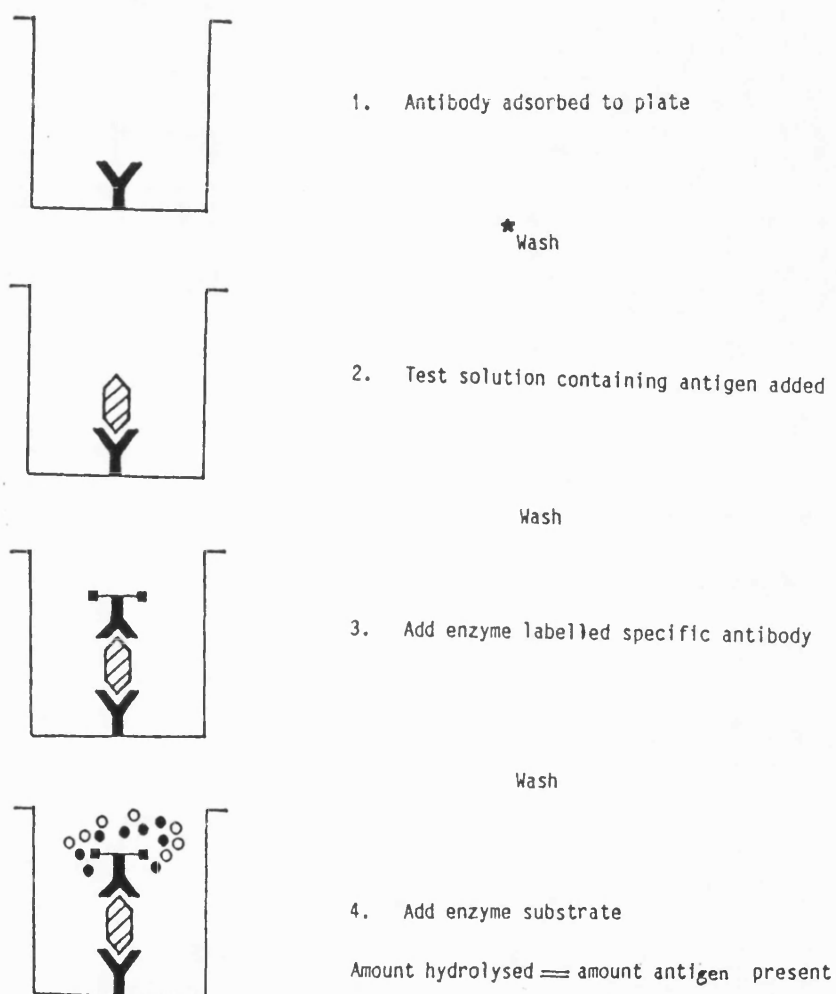
4.3. SETTING UP THE ELISA ASSAY.

First it was required to set up suitable ELISA assays for measurements of IgG and IgM in samples supernatants. The principle of such an assay is depicted in Fig. 4.2.

It was first necessary to consider choice of support, that is the type of ELISA plate, since although all plastics nonspecifically adsorb protein [8], different plastics do so to varying degrees. Flat bottomed polystyrene NUNC Immuno-I plates (Tissue Culture Services) were chosen since they show high protein adsorption but low inter and

FIG 4.2 Illustrating the double antibody ELISA used for quantifying IgG and IgM in culture supernatants

*Excess binding sites on the solid phase were blocked using PBS-0.5% BSA before proceeding to step 2.



KEY TO FIG



= Goat antihuman polyvalent immunoglobulin



= IgG/IgM containing culture supernatants/standards



= Affinity purified antihuman IgG/IgM alkaline phosphatase conjugate



= d-nitrophenyl phosphate (native and degraded)

intra plate variations, low background readings and are economical to buy.

ELISA plates were prepared by binding goat antihuman polyvalent Ig antiserum (a dilution of 1:200 in carbonate buffer, found to be optimal) and excess binding sites blocked by a one hour incubation at 37 °C with PBS-0.5% BSA and this was left in the ELISA plate wells. Any samples that required dilution were diluted in PBS-0.5% BSA. Thus all culture supernatants (containing 10% FCS) were prior diluted and double diluted in a protein containing solution, without producing an overall disturbance in the protein content of the system which might otherwise affect the way the antigen interacted with the various immunological reagents used in the ELISA.

Standard curves were set up using pooled normal human serum, negative for Rheumatoid factor (LATEX agglutination test), and diluted to 4000 ng/ml for both IgG and IgM concentrations such that with eleven doubling dilutions the standard curve ranged from 2000 to 2 ng/ml for both IgG and IgM.

Incubation times with sample supernatant or conjugate were strictly controlled, since background Ig levels were found to increase when antigen, and in particular conjugate, incubation times exceeded 1 hour at 37 °C. If a large batch of the ELISA plates was to be analysed, plates were coated with antigen and left in the fridge until all were ready and all were incubated for exactly 1 hour at 37 °C. Conjugate addition was staggered to allow an exact incubation time of 1 hour at 37 °C for each plate. Optimum dilution of TAGO conjugate for

the IgM ELISA was found to be 1:3000 and 1:1000 for SIGMA IgG conjugate.

ELISA sensitivity was determined by setting up very low concentrations of IgG and IgM in quadruplicate and absorbancy compared (one-tailed T-test) with results obtained when no IgG or IgM was present. Sensitivity was defined the lowest concentration of IgG or IgM which gave an absorbance value significantly different ($p < 0.05$) to that given in the absence of IgG or IgM. The ELISA method was found to be sensitive down to 4 ng/ml and 8 ng/ml for IgG (Fig. 4.3) and IgM respectively, however, in practice, because an edited standard curve (Fig. 4.4) was used for calculation of the sample concentration utilizing only the linear portion of the standard curve, the useful range for IgG is down to 16 ng/ml and for IgM down to 32 ng/ml (Figs. 4.5-4.6). The useful range extended up to approximately 500 ng/ml and 800 ng/ml for IgG and IgM respectively

The specificity of the respective ELISA was ensured by using:

- purified antihuman γ -specific alkaline phosphatase conjugate for the IgG ELISA;
- purified antihuman μ -specific alkaline phosphatase conjugate for the IgM ELISA.

Plates coated with purified myeloma IgG1 in the range 2 to 2000 ng/ml compared with those coated with pooled human serum containing all classes of IgG showed close parallelism using the same alkaline phosphatase γ -specific conjugate, showing that the same antigen was being detected (Fig. 4.7).

FIG. 4.3

UNEDITED VERSION OF IgG STANDARD CURVE

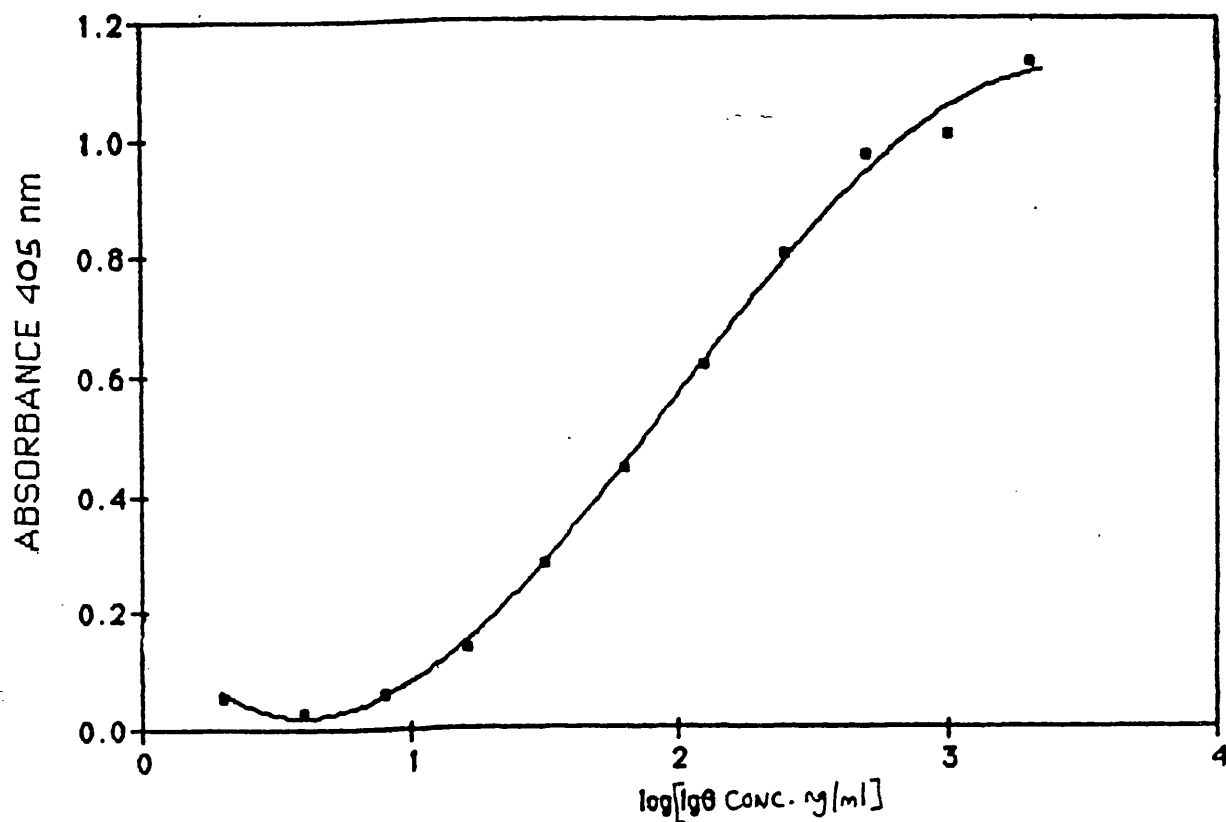


FIG. 4.4

EDITED VERSION OF IgG STANDARD CURVE

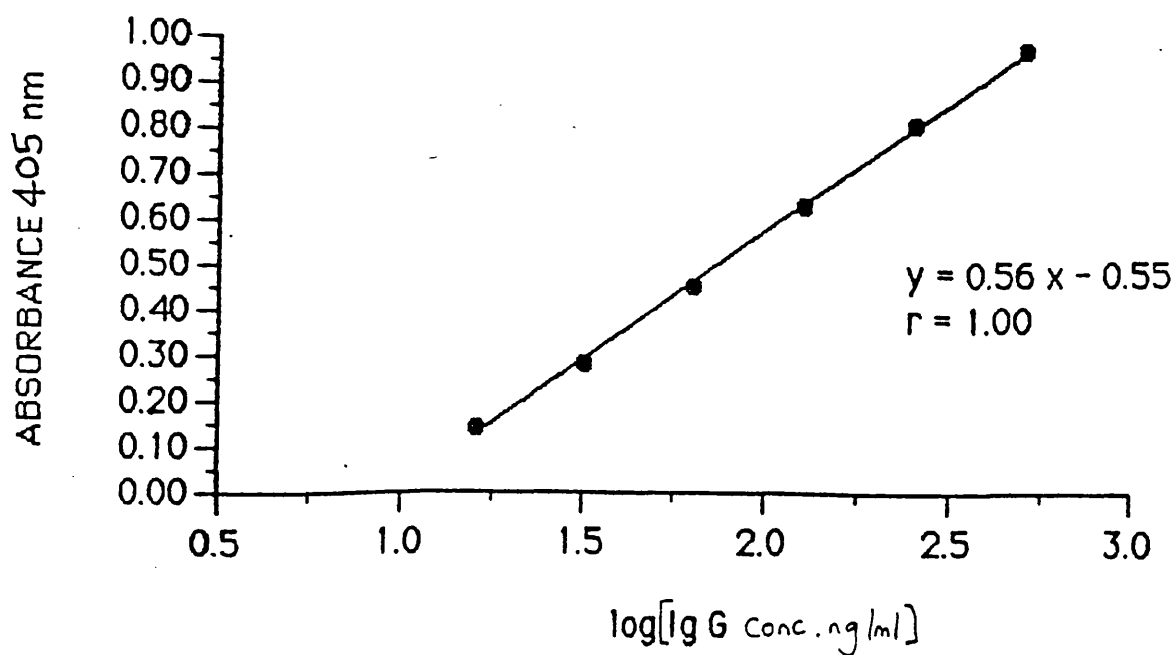
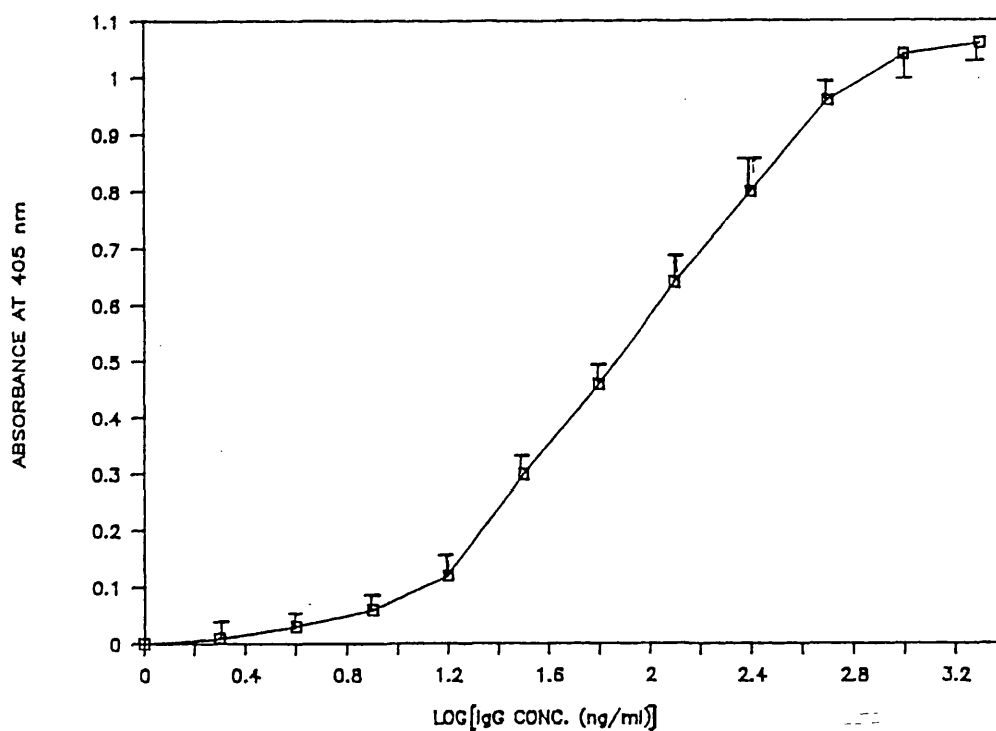


FIGURE 4.5

INTRAPLATE REPRODUCIBILITY FOR IgG

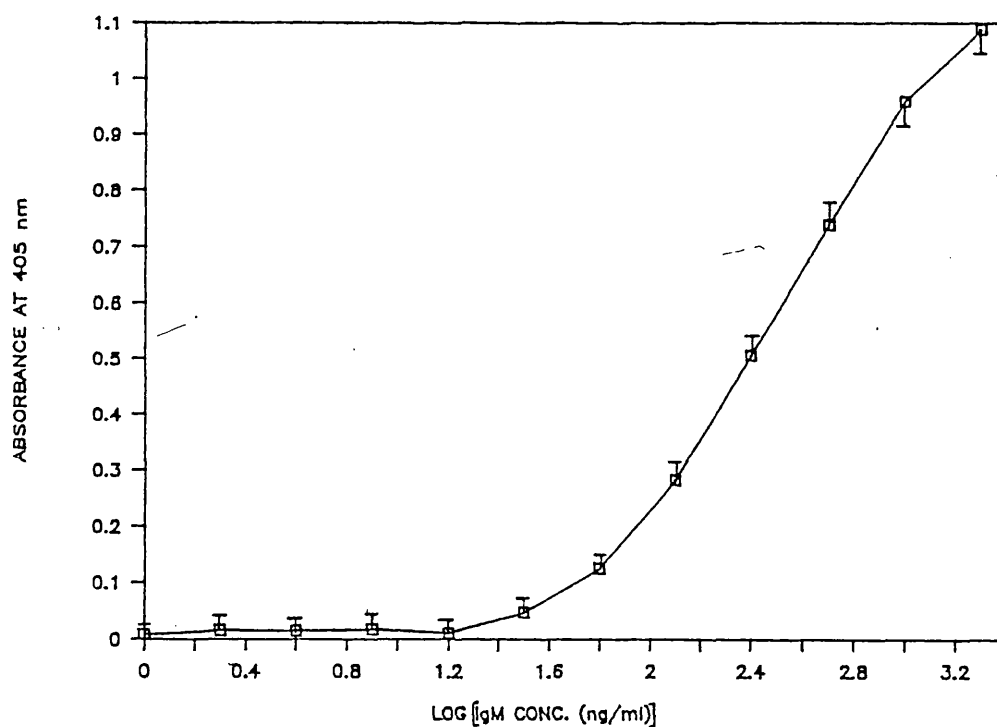


Data points represent mean of 8 IgG standard curves ± 1 SD.

At all points the coefficient of variation was less than 6%.

FIGURE 4.6

INTRAPLATE REPRODUCIBILITY FOR IgM



Data points represent mean of 8 IgM standard curves ± 1 SD.

At all points the coefficient of variation was less than 6%.

FIGURE 4.7 SPECIFICITY OF IgG ELISA

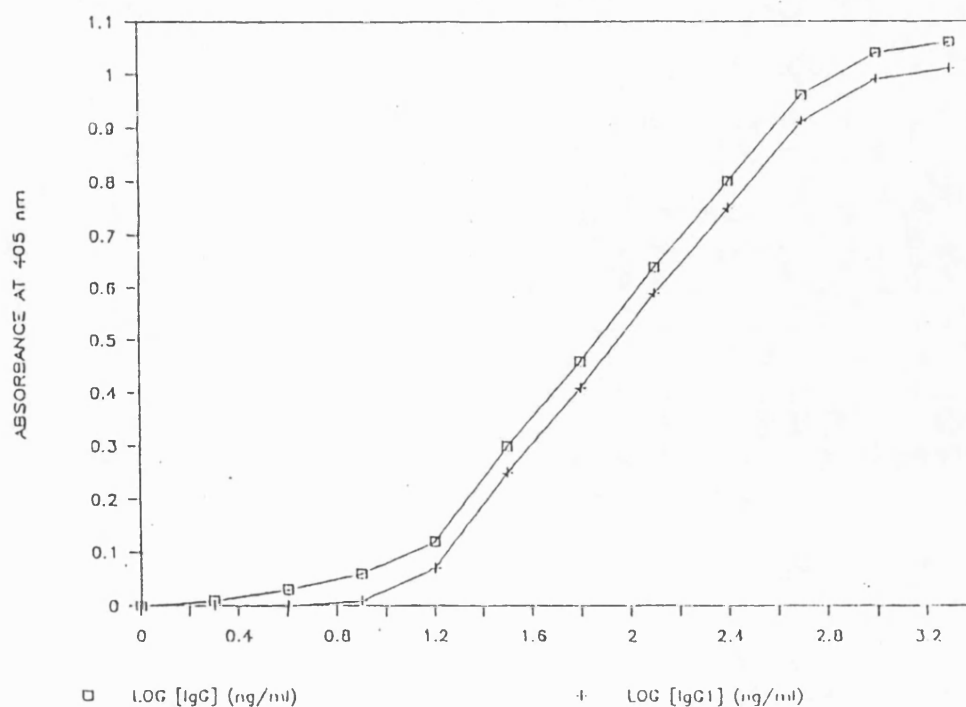
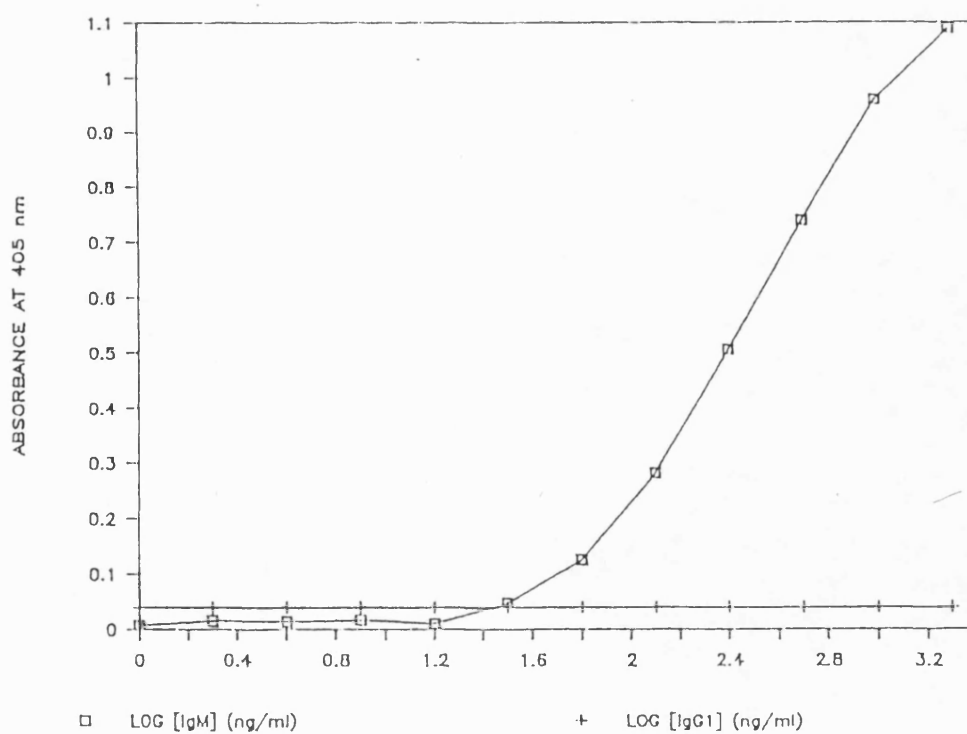


FIGURE 4.8 SPECIFICITY OF IgM ELISA



Data points represent mean for $n = 2$ observations.

SEM omitted for clarity.

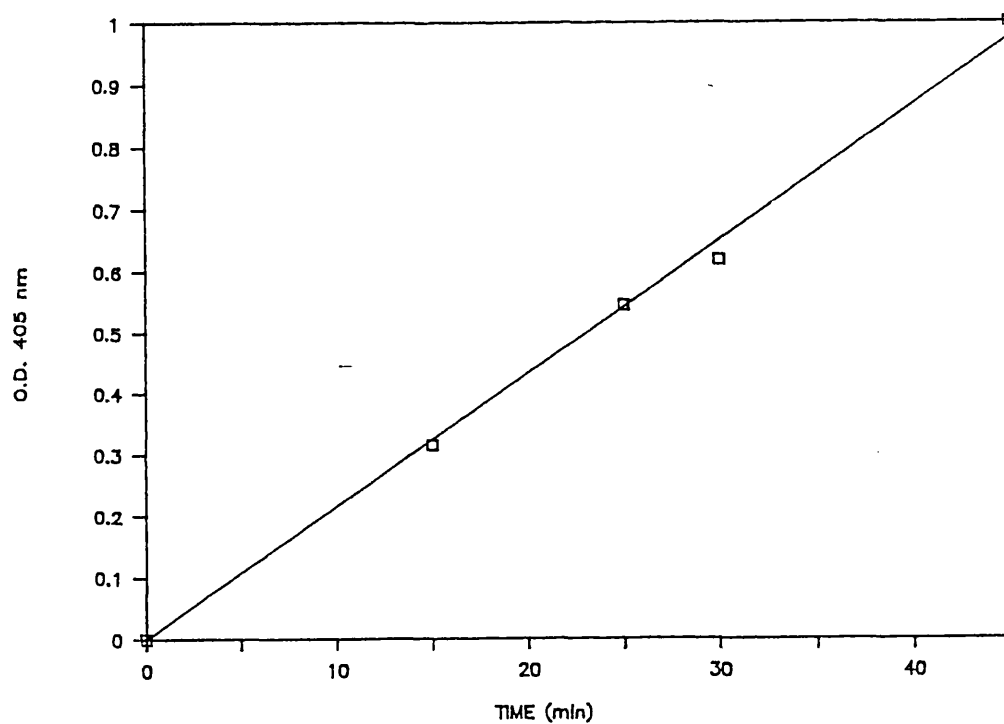
Antihuman μ -specific alkaline phosphatase was found not to bind to plates coated with purified IgG1 myeloma protein only to those coated with the IgM (IgG1 was used since it is simpler to prepare than purified total human IgG and its use can be justified since IgG1 represents the major subclass of IgG present in normal serum) (Fig. 4.8).

By putting a standard curve of IgG or IgM on 8 rows over the all ELISA plate, intraplate reproducibility was found to be excellent (with coefficients of variation for all concentrations being less than 6%) so long as the plate was tightly covered with "cling film" to prevent "edge effects" (Figs. 4.5 and 4.6). Interplate reproducibility (on different plates within a day or on different days) was equally good. Similar intra and inter plate coefficients of variation were found using sample supernatants (data not shown).

The degradation of substrate (1 mg/ml-para-nitrophenyl phosphate diluted in diethanolamine buffer) with time, as measured by an increase in absorbance at 405 nm showed a linear colour development (Figs. 4.9-4.10) and usually required 45 minutes at room temperature for development of the IgG standard curve or half an hour for the IgM standard curve. Termination of the reaction was in both cases when the top standard reached an absorbance of 1.0. Reaction times depended on the potency of the conjugate used and the ambient temperature, but in all cases the conjugates were sufficiently potent for the reaction to proceed at room temperature. To obtain meaningful results the ELISA system must be adjusted so that the reaction shows

FIGURE 4.9

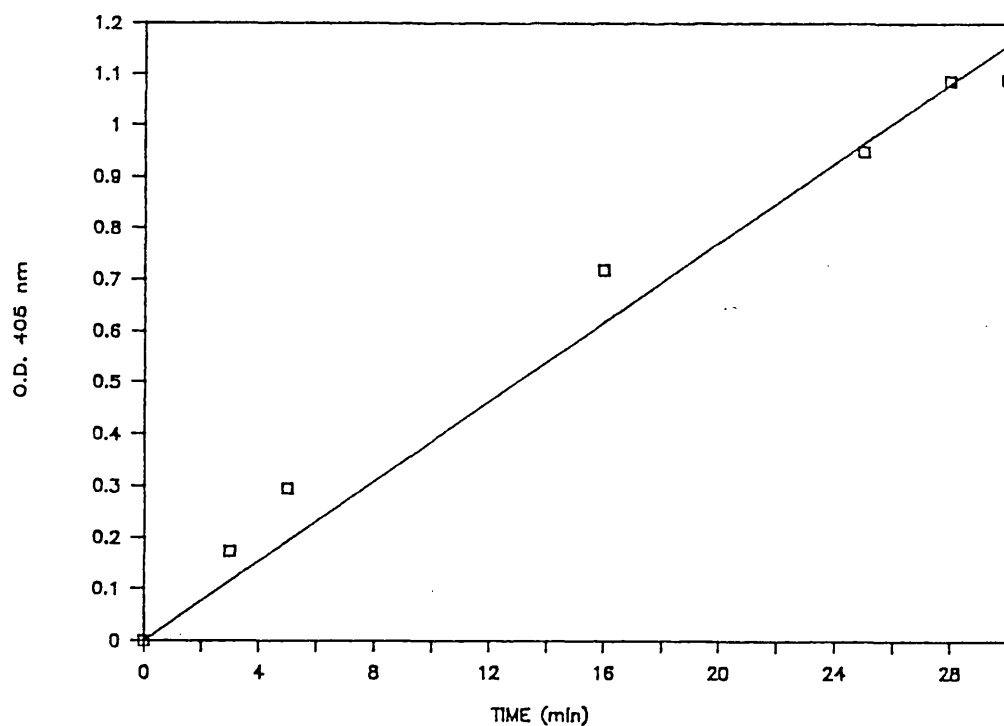
TIME COURSE OF TOP IgG STANDARD



Showing linearity of colour development of the IgG ELISA.

FIGURE 4.10

TIME COURSE OF TOP IgM STANDARD



Showing linearity of colour development of IgM ELISA.

O.D. = optical density.

zero order kinetics and is thus independent of substrate concentration (which must be in excess to saturate the enzyme) and only dependent on the enzyme concentration. In the presence of excess ELISA reagents (polyvalent, substrate and enzyme) rates of colour development will be dependent only on the amount of the enzyme present, which is directly proportional to the quantity of IgG and IgM attached to the polyvalent layer.

Standard curves of absorbancies against IgG or IgM concentration in ng/ml were constructed and immunoglobulin content in sample supernatants determined using edited standard curves, so that only the linear portion of the curve - i.e. the section obeying Beer-Lambert's law - was used. These values were then suitably multiplied by the dilution factors, and the average result from all readings that showed doubling out of immunoglobulins (that is the linear portion of sample standard curves) used for calculating the final immunoglobulin content in ng/ml of supernatant, results were finally expressed in terms of ng Ig/10⁶ PBMNC of supernatant.

4.4. EFFECT OF PHMPA ON IgG SYNTHESIS.

The ELISA systems were initially used to investigate the thiol dependence of IgG or IgM production.

Initial experiments were set up to find the optimal cell density and pokeweed mitogen dilution to use for this experiment. Table 4.2 shows a cell density of 1×10^6 /ml and a pokeweed mitogen dilution of 1:200 to be optimal, for maximal IgG synthesis (similar results were

TABLE 4.2

EFFECT OF INCREASING CELL CONCENTRATION ON IgG SYNTHESIS (ng/ml) BY
 NORMAL PWM STIMULATED PBMNC, DURING A 7 DAY CULTURE PERIOD. RESULTS
 ARE EXPRESSED AS MEAN \pm SEM (IN BRACKETS).

[PWM]	CELL CONCENTRATION ($\times 10^6$ /ml)			
	0.25	0.50	1.00	2.00
1/20	224 (-) n=1	1076 (619) n=5	2073 (986) n=4	2159 (808) n=12
1/200	384 (-) n=1	2377 (711) n=6	3419 (822) n=14	3015 (767) n=16
1/400			2553 (557) n=9	
1/1,000			2382 (508) n=9	
1/2,000	224 (-) n=1	1431 (515) n=4		1261 (306) n=13
1/10,000	54 (-) n=1	657 (123) n=5	527 (21) n=2	827 (334) n=11

obtained for IgM but data are not shown).

The kinetics of IgG and IgM production were then investigated in various patient groups and similar kinetics were found in normals, RA NSAID, RA-DPEN/AU, AS, PSA and SLE patients. In all cases background immunoglobulin production rose very slightly over the 21 days culture period and 1:200 dilution pokeweed mitogen stimulated immunoglobulin production was shown to remain very low for the first 5 days in culture, but then to rise exponentially between days 5 to 10 and a much slower rise in immunoglobulin production occurred from days 10 to 21 as is shown in Figs. 4.11 and 4.12 for a normal individual.

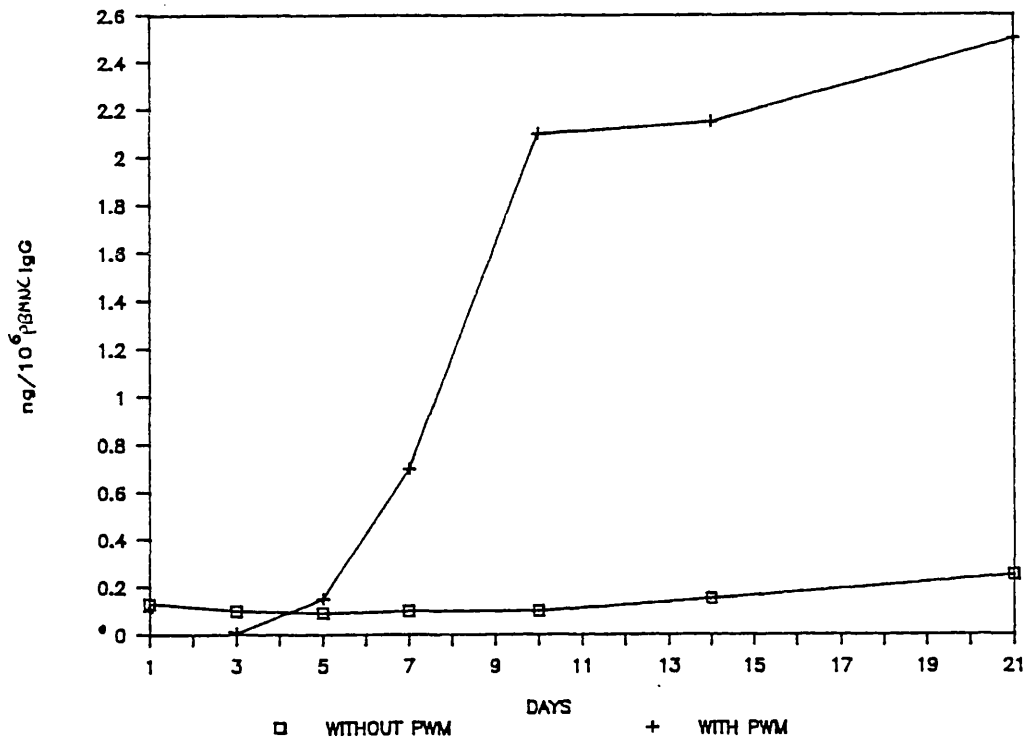
Day 1 indicating day of culture set up and day 21 that of culture termination.

A culture time of 8 days was chosen in all subsequent experiments since it represented a convenient culture period and was on the exponentially rising phase of IgG or IgM production.

Normal PBMNC were diluted to $5 \times 10^6/\text{ml}$ in PBSC or RPMI and treated for 1 hour at 37°C with $50\ \mu\text{M}$ PHMPA (an irreversible surface thiol blocking agent) and subsequent IgG production measured; these choices of cell density, time and temperature of incubation, and PHMPA concentration were shown to be optimal (see below). It was found that more inhibition of IgG synthesis was obtained in a PBSC medium $\text{ID}_{50} = 12\ \mu\text{M}$ compared to an $\text{ID}_{50} = 25\ \mu\text{M}$ in RPMI (see Fig. 4.13), probably because effective PHMPA concentration were reduced by binding to free thiol groups on albumin present in the FCS component of the culture medium so in all further experiments, cells were

FIGURE 4.11

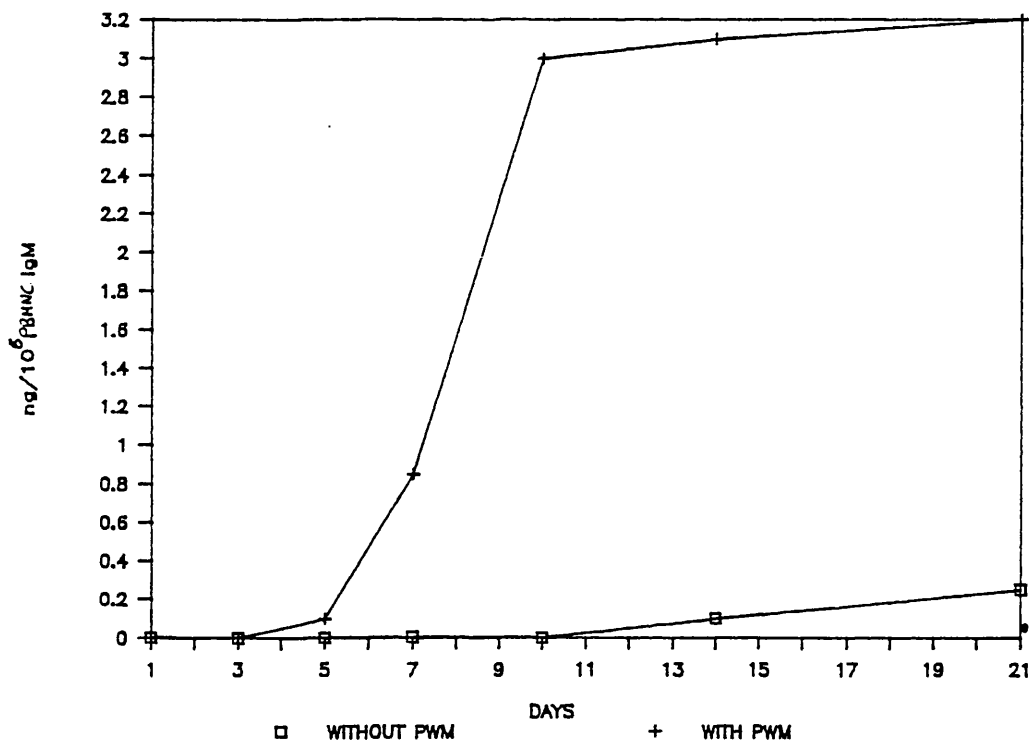
IgG PROD.N IN A NORMAL SUBJECT



Showing kinetics of IgG production over a 21 day culture period with or without pokeweed mitogen (PWM). Each point represents mean of n = 2 observations. SEM were omitted for clarity.

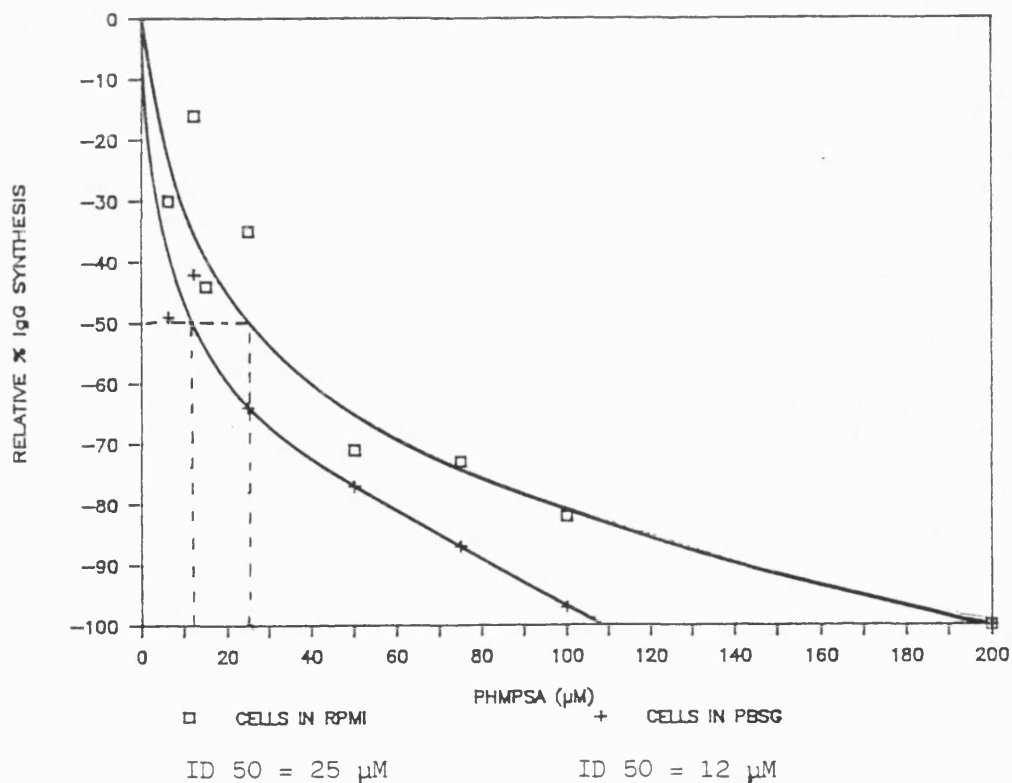
FIGURE 4.12

IgM PRODUCTION IN A NORMAL SUBJECT



Showing kinetics of IgM production over a 21 day culture period with or without pokeweed mitogen (PWM). Each point represents mean of $n = 2$ observations, SEM were omitted for clarity.

FIG. 4.13 EFFECT OF COMPOSITION OF MEDIUM IN WHICH CELLS ARE INCUBATED WITH PHMPA ON SUBSEQUENT RELATIVE % IgG SYNTHESIS



Lines represent mean ($n = 4$) relative % IgG synthesis following a 1 hour preincubation at 37 °C with various concentrations of PHMPA when added to cells at 5×10^6 /ml in 2 different media, then washed and resuspended in culture medium at 1×10^6 /ml, grown with 1:200 PWM for a week. Cell viability was not effected by prior treatment with PHMPA - as assessed by ethidium bromide after the 1 week culture period.

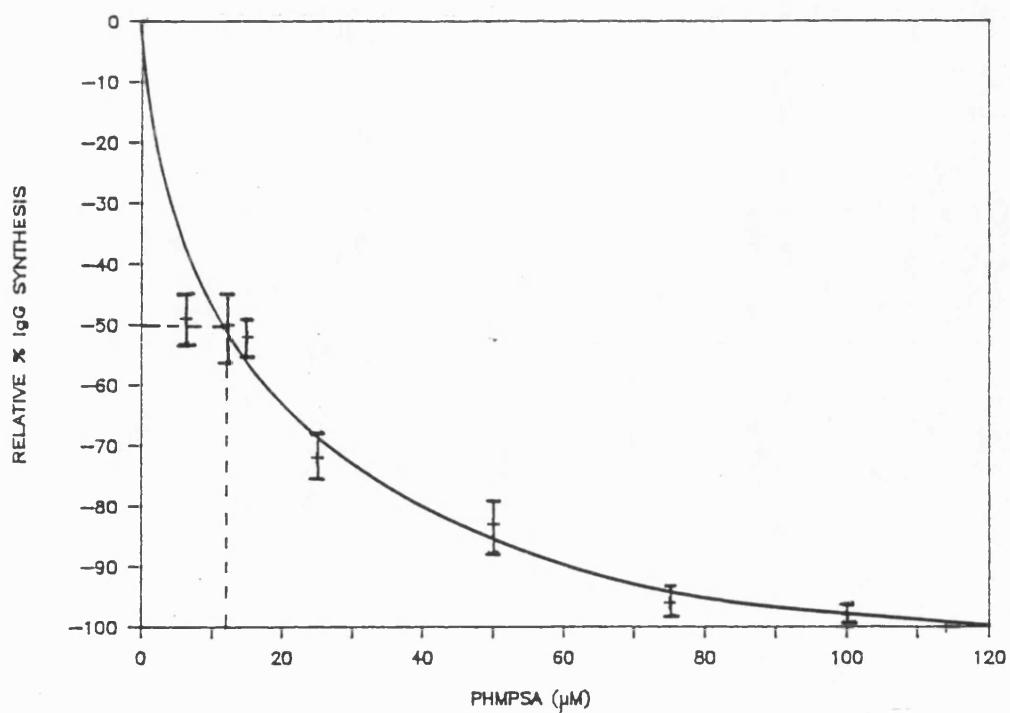
incubated with PHMPSA only in PBSG media. A larger experiment was carried out with $N = 7$ normals and results shown in Fig. 4.14., again showing ID50 to be $12 \mu\text{M}$ PHMPSA.

Relative percent IgG synthesis was found to be dependent on the density of cells incubated with a particular PHMPSA concentration since cells at $1 \times 10^6/\text{ml}$ showed an ID50 of $7.5 \mu\text{M}$ with as little as $15 \mu\text{M}$ PHMPSA causing a 100% suppression of IgG synthesis. Cells at $2.5 \times 10^6/\text{ml}$ showed an ID50 of $10 \mu\text{M}$ with $50 \mu\text{M}$ PHMPSA giving 90% suppression of IgG synthesis. Cells at $5 \times 10^6/\text{ml}$ showed an ID50 of $12 \mu\text{M}$ with $50 \mu\text{M}$ PHMPSA giving 85% suppression of IgG synthesis. Cells at $10 \times 10^6/\text{ml}$ showed an ID50 of $30 \mu\text{M}$ with $50 \mu\text{M}$ of PHMPSA showing approximately 70% suppression of IgG synthesis. In all cases $100 \mu\text{M}$ PHMPSA showed approximately 100% suppression of IgG synthesis but little effect on cell viability (Figs. 4.15). IgM production showed a similar thiol dependence (data not shown). A 60 minute incubation at 37°C was chosen to allow significant immunoglobulin inhibition (Table 4.3) without appreciably affecting cell viability (Table 4.4). A 1 hour incubation at 37°C itself reduced immunoglobulin synthesis due to preferential loss of B cells with time (Table 4.5), but cell viability is not reduced significantly if vessel lids are left loose for CO_2/O_2 exchange to occur (Table 4.6).

4.5. COMPARISON OF IgG AND IgM SYNTHESIS IN VARIOUS PATIENT GROUPS AND NORMALS.

In the light of the thiol dependence of immunoglobulin production it was decided to investigate IgG and IgM production in various

FIG. 4.14 EFFECT OF PREINCUBATION OF PBMNC IN PBSG WITH PHMPSA
ON SUBSEQUENT RELATIVE IgG SYNTHESIS



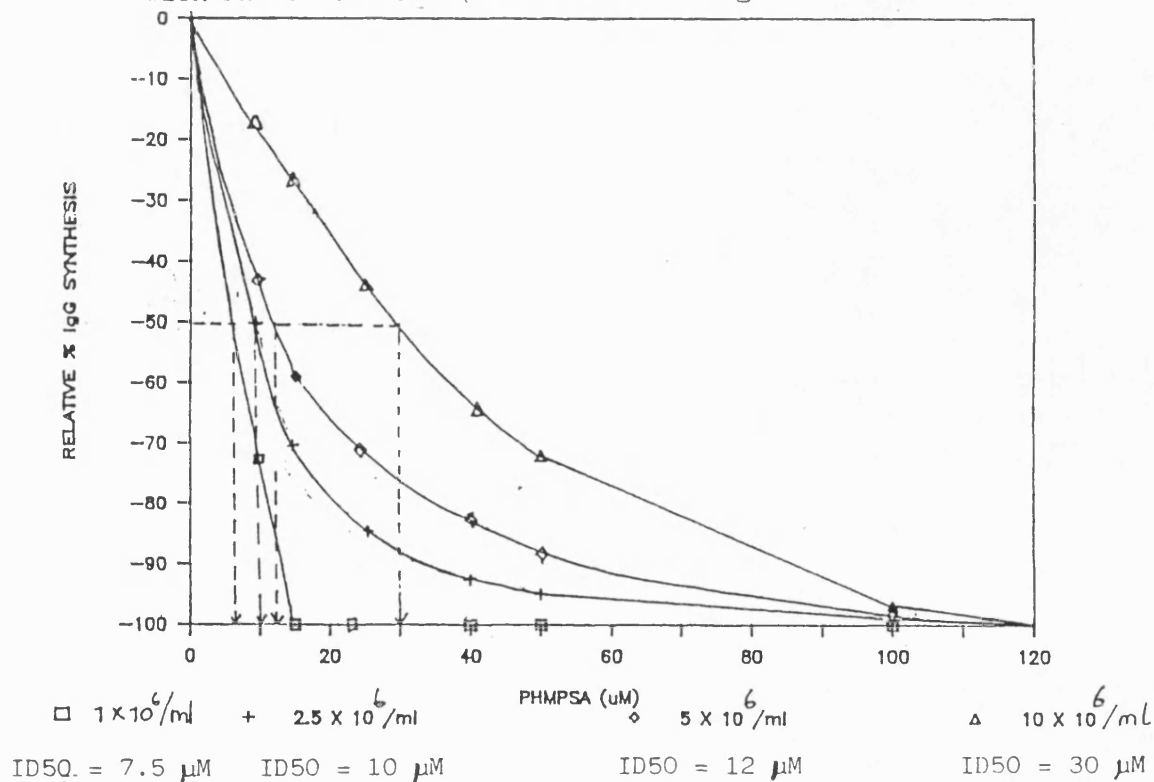
ID50 = 12 μ M

Line represents mean \pm SEM of $n = 7$ individuals.

Cells at 5×10^6 /ml in PBSG were incubated with various concentrations of PHMPSA for 1 hour at 37 °C then washed and cultured for 1 week in medium with 1:200 PWM.

FIG. 4.15 EFFECT OF CELL DENSITY IN WHICH CELLS ARE INCUBATED

WITH PHMPA ON SUBSEQUENT RELATIVE % IgG SYNTHESIS



Where ID_{50} is the concentration of PHMPA causing 50 % inhibition of IgG synthesis.

Each line represents mean of $n = 3$ individuals (SEM omitted for clarity).

TABLE 4.3

TIME COURSE OF % INHIBITION OF IgG PRODUCTION USING 50 μ M PHMPA, PREVIOUSLY SHOWN TO GIVE ALMOST MAXIMUM SUPPRESSION OF IgG SYNTHESIS WITHOUT AFFECTING CELL VIABILITY RESULTS ARE EXPRESSED AS MEAN \pm SEM (n = 4).

TIME (min)	RELATIVE % IgG SYNTHESIS
0	0
15	50 \pm 16 %
30	60 \pm 11 %
60	72 \pm 12 %
90	82 \pm 12 %
60 (NO PHMPA)	0

TABLE 4.4

VIABILITY OF CELLS INCUBATED IN ABSENCE OR PRESENCE OF 50 μ M PHMPA FOR VARIOUS LENGTHS OF TIME.

TIME (min)	NO PHMPA	WITH PHMPA
0	100 %	100 %
15	100 %	98 %
30	100 %	98 %
60	99 %	97 %
90	97%	94 %
		↓
		viability unacceptably low

TABLE 4.5

EFFECT OF 1 HOUR INCUBATION AT 37 °C ON IgG PRODUCTION, CELLS EXPOSED TO 5% CO₂/AIR THROUGHOUT THE INCUBATION PERIOD. RESULTS ARE EXPRESSED AS ng/10⁶PBMNC IgG PRODUCED, HAVING SUBTRACTED BKG LEVELS.

SUBJECT No.	TIME 0'	TIME 60'
1	4238	3295
2	1781	1100
3	595	577

TABLE 4.6

COMPARISON OF INCUBATIONS WITH/WITHOUT VARIOUS PHMPA CONCENTRATIONS AT 37 °C IN A WATER BATH WITH THE TUBE LID TIGHTLY CLOSED OR WITH LOOSE LIDS ALLOWING GASEOUS EXCHANGE TO TAKE PLACE, ON % VIABLE CELL REMAINING.

PHMPA (μM)	CLOSED LID (n = 2)	LOOSE LID (n = 4)
6.25	94 %	98 %
12.50	91 %	98 %
25.00	86 %	98 %
50.00	77 %	97 %
100.00	74 %	90 %
NO PHMPA	95 %	99 %

patient groups, to see if reduced serum thiol levels and therefore presumably reduced cell surface thiol levels are associated with defective immunoglobulin synthesis (Table 4.7 summarises the patient population used throughout the remaining section of the thesis).

Since no differences were observed in RA patients +/- RF all such patients were grouped together. Median PWM stimulated IgG production (Fig. 4.16) in RA-DPEN and AS/PSA patients and normals was found to be not dissimilar; however IgG production in RA NSAID patients was significantly ($p < 0.005$) decreased compared to normals. This is partly due to the significantly raised spontaneous IgG synthesis ($p < 0.05$ compared to normals) (Table 4.8): in fact it was often 2 to 3 times higher, which could be due to defective immunoregulation, in particular defects in suppressor lymphocytes, preventing "switching off" of IgG production.

Fig. 4.17 shows median PWM stimulated IgM production in normals and AS/PSA and RA D-PEN patients was not dissimilar; however it was significantly reduced in RA NSAID patients ($p < 0.05$). The reduced IgM levels here were not the result of raised spontaneous IgM production since this was not significantly different in all 4 groups (Table 4.9), although the trend was for greater spontaneous IgM synthesis in RA NSAID, possibly reflecting defective suppressor lymphocyte systems.

In the light of the PHMPA experiment showing the thiol dependence of the immunoglobulin production, it was wondered whether the defective PWM stimulated immunoglobulin synthesis observed in RA NSAID patients was due to thiol oxidation of functionally important

TABLE 4.7

DESCRIPTIVE SUMMARY OF ALL PATIENT POPULATION USED THROUGHOUT THE THESIS. DATA EXPRESSED AS MEDIAN (RANGE IN BRACKETS).

	RA NSAID	RA D-PEN/GOLD	AS/PSA	SLE
NUMBER	26	17 (12 D-PEN AND 5 GOLD)	17 (13 AS AND 4 PSA)	7
M:F	8:18	6:11	9:8	0:7
AGE (years)	62 (19 - 82)	48 (21 - 76)	46 (27 - 79)	57 (49 - 65)
DISEASE DURATION § (MONTHS)	6.5 (1 - 42)	6.0 (1 - 28)	16 (1 - 38)	6 (5 - 10)
TREATMENT DURATION (MONTHS)	ALL FOR MORE THAN 6 MONTHS	8 (4 - 96)	-	ALL FOR MORE THAN 6 MONTHS
DRUGS DOSAGE (mg/day)	NSAIDs : VARIABLE	D-PEN 500 (125 - 625) GOLD [AVG./DAY] 3.7 (0.5 - 7.1)	14 ON NSAIDs WHEN NEEDED (VARIABLE DOSE) 3 ON NO Rx	2 ON HYDROXY- CLOPROQUINE (400)
ACTIVE * PERIPHERAL SYNOVITIS	SEVERE 26 MODERATE - NOT PRESENT -	SEVERE 3 MODERATE 13 NOT PRESENT 1	SEVERE - MODERATE 12 NOT PRESENT 5	SEVERE - MODERATE - NOT PRESENT 7
EXTRA- ARTICULAR DISEASE	14/26	1/17	1/17	1/7
R.F. (IU/ML)	97.5 (0 - 620)	101 (0 - 2084)	-	25 (0 - 140)
EMS (min)	90 (23 - 180)	105 (15 - 210)	-	-
CRP (g/l) §	0.045 (0.01 - 0.204)	0.027 (0.01 - 0.064)	0.01 (0.01 - 0.011)	0.038 (0.015 - 0.062)
VISCOSITY (cp)	1.88 (1.54 - 2.29)	1.9 (1.5 - 2.18)	1.72 (1.61 - 1.85)	1.7 (1.68 - 1.72)
WBC (10 ⁹ /l)	7.3 (4.1 - 15.9)	7.7 (4.8 - 10.2)	6.6 (4.8 - 8.4)	7.0 (6.3 - 7.6)
PLTS (10 ⁹ /l)	358 (198 - 626)	351 (212 - 534)	311 (253 - 524)	313 (293 - 333)
LYMPHOCYTES (10 ⁹ /l)	1.6 (0.8 - 3.6)	1.6 (0.8 - 2.0)	1.7 (0.9 - 2.7)	1.6 (0.9 - 2.3)
Hb (g/dl)	11.7 (8.1 - 14.9)	13.1 (10.3 - 16.0)	13.6 (11.6 - 16.2)	13.2 (12.1 - 14.3)
HLA B27 +ve			5/17 (29%)	

§ = < 1 taken as 1

* = as assessed by referring physician

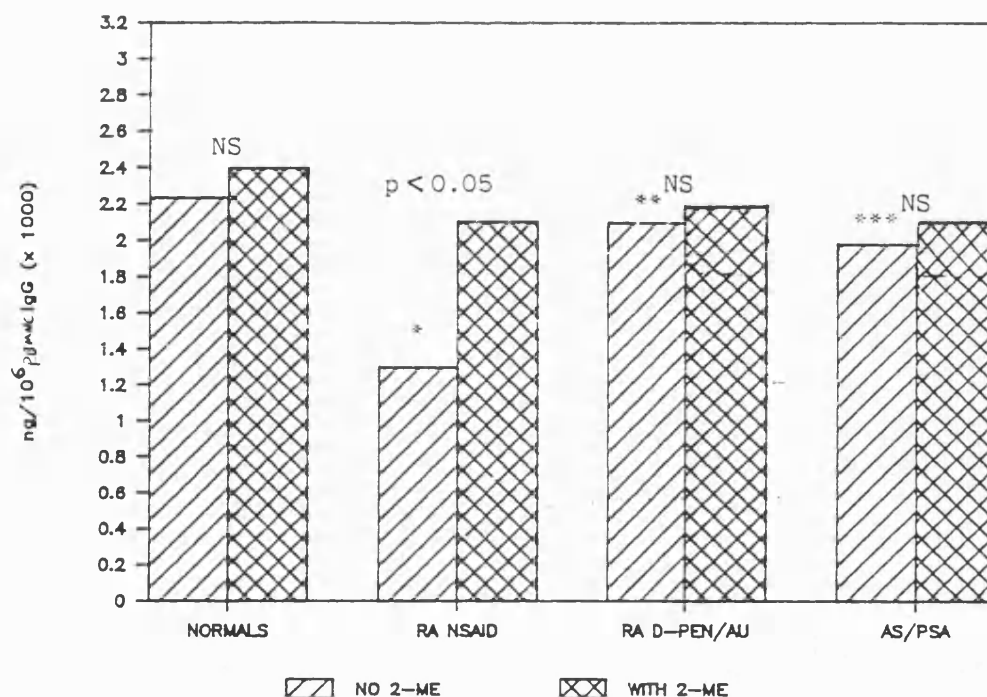
§ = < 0.01 taken as 0.01

LABORATORY RANGES FOR NORMAL HEALTHY CONTROLS

RF	0-40 IU/ml (latex agglutination test)
EMS	0 minutes
ESR	>15 mm/h (males) >20 mm/h (females)
CRP	<0.01 g/l
VISCOSITY	1.5-1.72 cp
WBC	4-11 x 10 ⁹ /l
PLTS	150-450 x 10 ⁹ /l
LØ	1.5-4.0 x 10 ⁹ /l
Hb	13.5-17.0 g/dl (males) 11.5-15.5 g/dl (females)
SERUM -SH LEVELS	350-580 µmol/l
IgA	1.5-4.0 g/l (Nephelometry)
IgG	8-17 g/l (Nephelometry)
IgM	0.6-2.0 g/l (Nephelometry)

TABLE 4.7 (continued)

FIG. 4.16 EFFECT OF PREINCUBATION WITH 2-ME ON SUBSEQUENT IgG PRODUCTION
IN VARIOUS PATIENT GROUPS



By Mann-Whitney U-test:

* = $p < 0.005$ vs. IgG production in absence of 2-ME in normals.

** = NS vs. IgG production in absence of 2-Me in normals.

*** = NS vs. IgG production in absence of 2-ME in normals.

Paired comparisons within each patient group were carried out by Wilcoxon signed rank test.

Results represent median IgG production (1:200 PWM - BKG) for $n = 10$

individuals in each group; IQR were omitted for clarity.

Cells were preincubated at 5×10^6 /ml with 50 μ M 2-ME for 1 hour at 37 °C, washed and then cultured with 1:200 PWM for 1 week.

TABLE 4.8

BACKGROUND IgG SYNTHESIS IN VARIOUS PATIENT GROUPS. RESULTS EXPRESSED AS
ng/10⁶ PBMNC

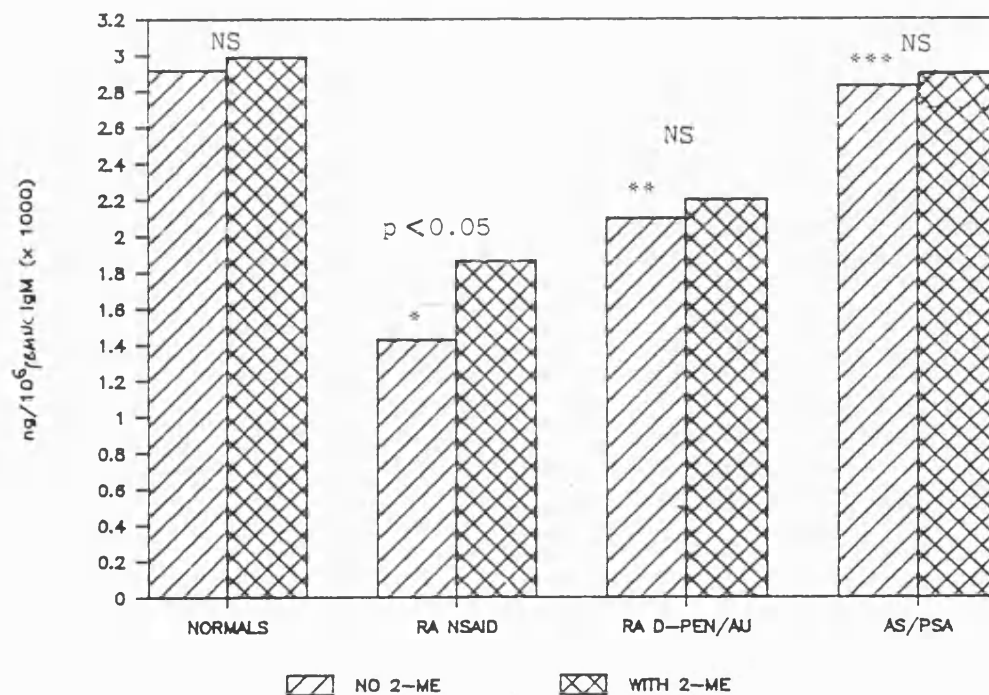
	NORMALS	RA NSAID	RA D-PEN	AS/PSA
NO 2-ME	239	450	238	250
WITH 2-ME	138	500	205	225

TABLE 4.9

BACKGROUND IgM SYNTHESIS IN VARIOUS PATIENT GROUPS. RESULTS EXPRESSED
AS ng/10⁶ PBMNC

	NORMALS	RA NSAID	RA D-PEN	AS/PSA
NO 2-ME	180	217	190	145
WITH 2-ME	118	199	105	145

FIG. 4.17 EFFECT OF PREINCUBATION WITH 2-ME ON SUBSEQUENT IgM PRODUCTION
IN VARIOUS PATIENT GROUPS



By Mann-Whitney U-test:

* = $p < 0.05$ vs. IgM production in absence of 2-ME in normals.

** = NS vs. IgM production in absence of 2-ME in normals.

*** = NS vs. IgM production in absence of 2-ME in normals.

Paired comparison within each group were carried out by Wilcoxon signed rank test.

Results represent median IgM production (1:200 PWM - BKG) for $n = 8$ individuals in each group; IQR were omitted for clarity.

Cells were preincubated at 5×10^6 /ml with 50 μ M 2-ME for 1 hour at 37 °C, washed and then cultured with 1:200 PWM for 1 week.

cell surface thiol groups and thus whether the situation could be rectified in vitro by incubation of PBMNC with 2-Mercaptoethanol (2-ME), a thiol reducing agent.

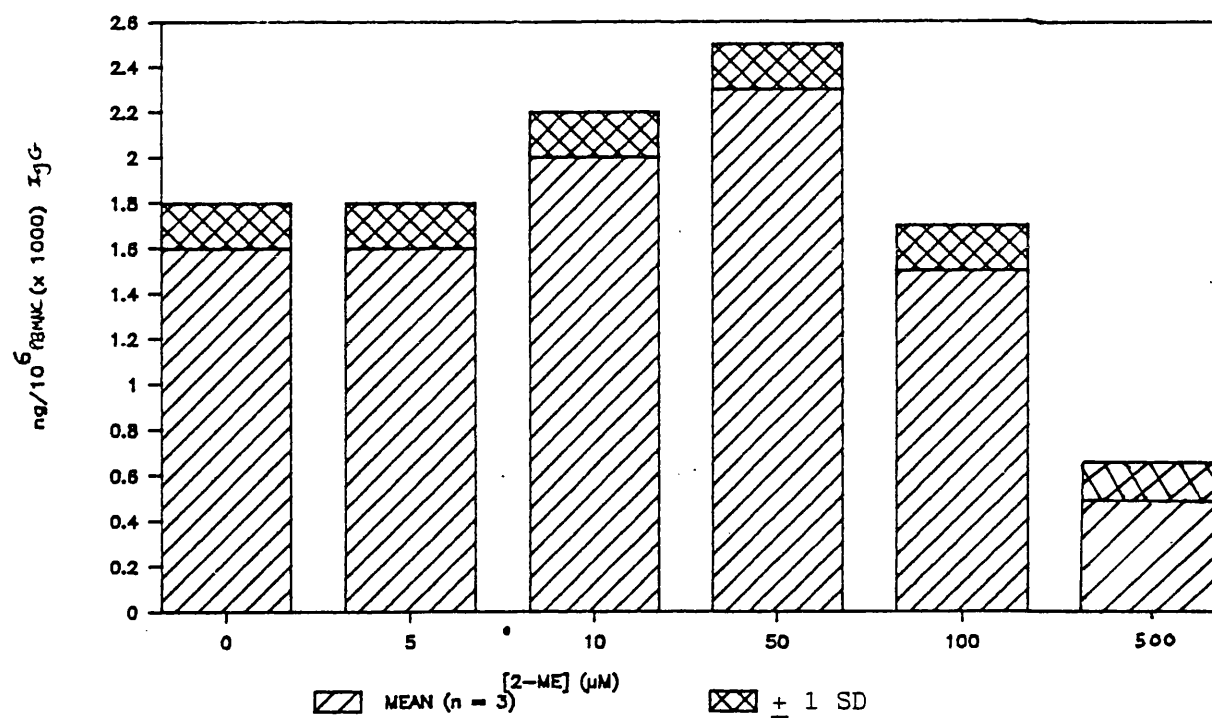
4.6. EFFECT OF 2-ME ON IgG AND IgM SYNTHESIS IN VARIOUS PATIENT GROUPS AND NORMALS.

In preliminary experiments it was found that preincubation of various concentrations of 2-ME for 1 hour with PBMNC prior to an 8 day PWM stimulated culture period did increase IgG synthesis in N = 3 RA NSAID patients and the optimum 2-ME concentration to utilise without affecting cell viability was 50 μ M, since at 500 μ M there was 70% cell death (Fig. 4.18); a similar concentration was optimal for IgM synthesis (data not shown). Leaving 2-ME in cultures for 8 days had a similar effect (data not shown). The effect of preincubation with 50 μ M 2-ME on PWM stimulated IgG synthesis was subsequently studied in a larger group of patients and compared to its effects on other patient groups (Fig. 4.16).

2-ME significantly improved ($p < 0.05$) IgG production only in RA NSAID, having a minor but not significant effect on normals, RA-DPEN and AS/PSA patients; similar normalization of immunoglobulin production was found for IgM synthesis in RA NSAID (Fig. 4.17), with mild but non significant effects on normal, RA D-PEN, AS/PSA subjects.

The hypothesis under investigation in this project is that defective immunoregulation seen in RA NSAID is due to oxidation of functionally important cell surface thiol groups and that this

FIG. 4.18
OPTIMUM 2-ME FOR IgG SYNTHESIS



The optimum 2-ME concentration to stimulate IgG production in RA NSAID
was found to be 50 μM.

Viability at 0, 5, 10 μM was = 99%

Viability at 50, 100 was = 95%

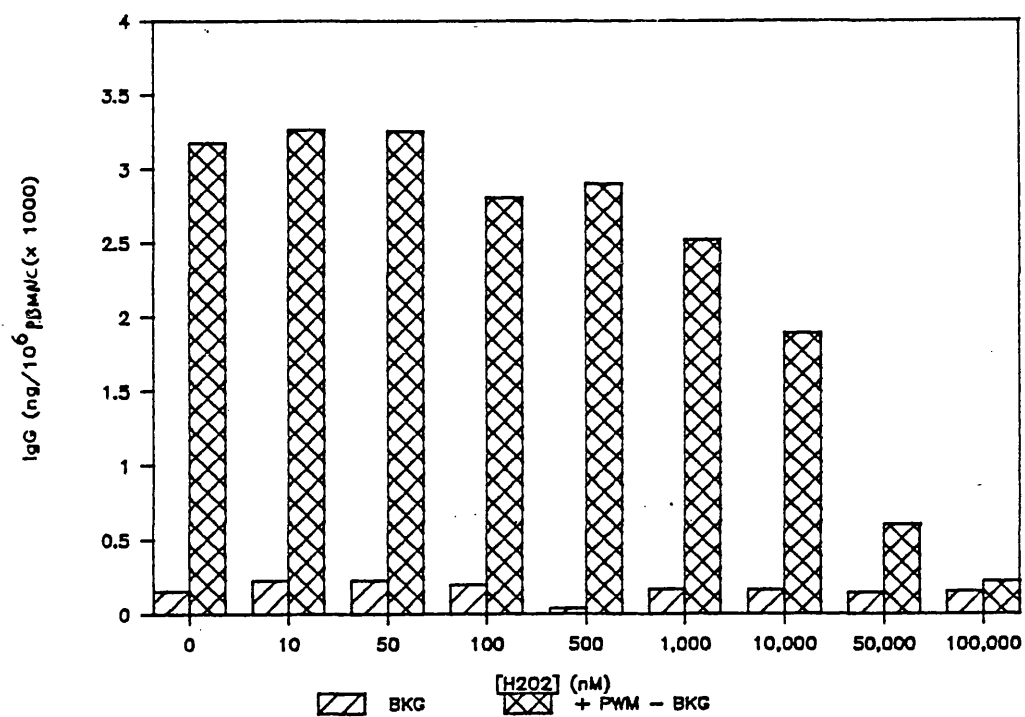
Viability at 500 μM was = 30%

oxidation is primarily the result of the highly reactive oxygen-free radicals species produced at sites of inflammation, one such species being H_2O_2 . Such molecules cause free -SH groups to be oxidized to form disulphide bonds with neighbouring thiol groups either within the same protein molecule, called intramolecular S-S bonds, or between small thiol-containing molecules in the serum e.g. cysteine or glutathione to form intermolecular S-S bonds.

4.7. EFFECT OF H_2O_2 ON IgG SYNTHESIS.

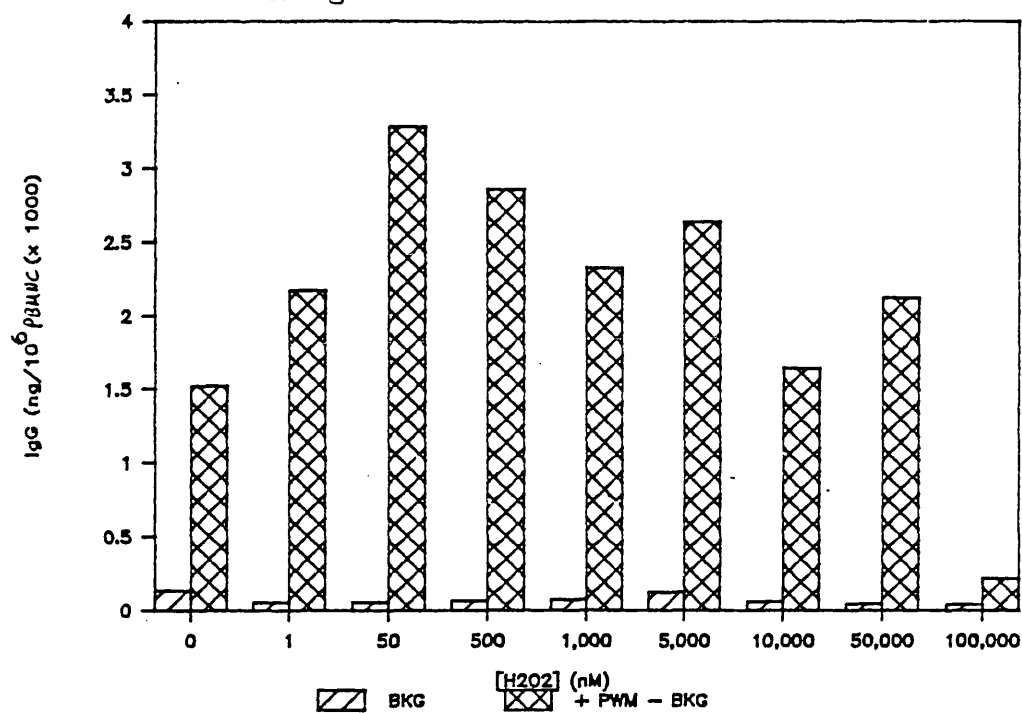
In an attempt to more precisely mimic the in vivo situation, rather than using the non-physiological thiol blocking agent PHMPA which probably blocks free -SH groups by binding to the sulphur atom with its Hg moiety, it was decided to investigate the effect of various doses of H_2O_2 left in culture on IgG synthesis (Fig. 4.19). The effect of H_2O_2 paralleled that using PHMPA, i.e. it inhibited IgG synthesis. In normals IgG synthesis fell significantly only after $10 \mu M$ H_2O_2 but cell viabilities were inhibited by 50% at this concentration and IgG synthesis was abolished at $50 \mu M$ H_2O_2 - not surprisingly since cell viability was obliterated at this concentration. There was a slight stimulation of IgG synthesis at very low H_2O_2 concentrations (10 to 50 nM) seen both in PWM stimulated and background cultures, indicating a slight mitogenic effect of H_2O_2 alone. Similar trends were found in RA-DPEN (Fig. 4.20), AS/PSA patients (Fig. 4.21) and RA NSAID patients (Fig. 4.22). There was some evidence to suggest that RA NSAID patients may be slightly more sensitive to further thiol oxidation of any remaining unoxidised thiol

FIG. 4.19 EFFECT OF H_2O_2 LEFT IN CULTURE
ON IgG PRODUCTION IN NORMAL SUBJECTS



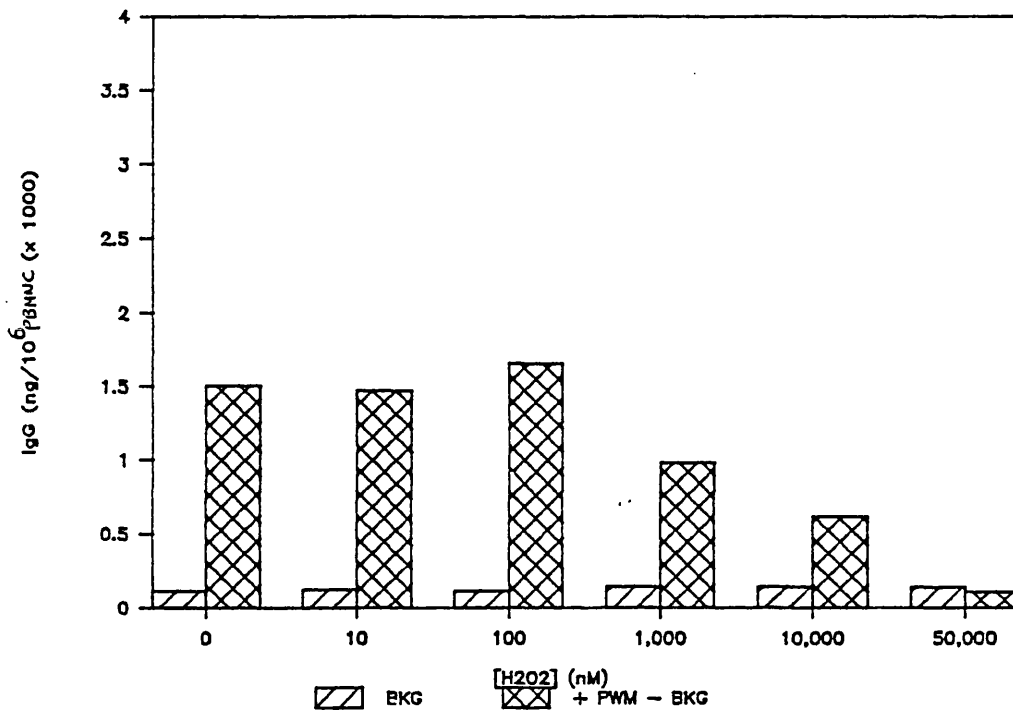
Showing mean IgG production with and without H_2O_2 (with and without 1:200 PWM) in $n = 13$ normal subjects. SEM were omitted for clarity.

FIG.4.20 EFFECT OF H_2O_2 LEFT IN CULTURE
ON IgG PRODUCTION IN RA D-PEN PATIENTS



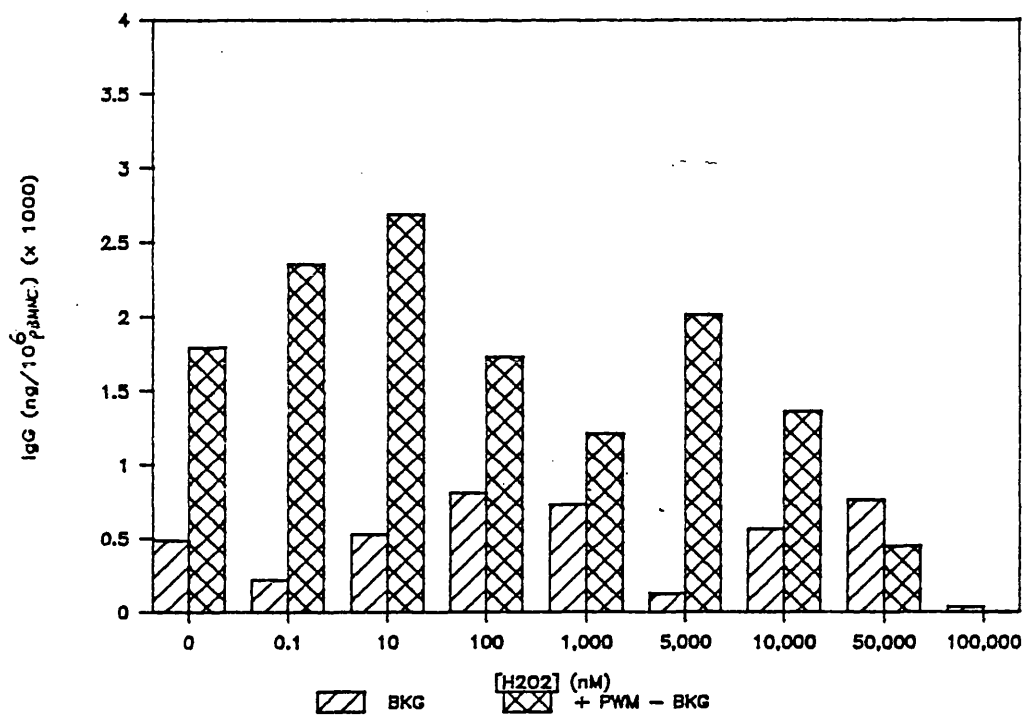
Showing mean IgG production with and without H_2O_2 (with and without 1:200 PWM) in n = 7 individuals; SEM were omitted for clarity.

FIG.4.21 EFFECT OF H_2O_2 LEFT IN CULTURE
ON IgG PRODUCTION IN AS/PSA PATIENTS



Showing mean IgG production with and without H_2O_2 (with and without 1:200 PWM) in n = 13 individuals; SEM were omitted for clarity.

FIG. 4.22 EFFECT OF H_2O_2 LEFT IN CULTURE
ON IgG PRODUCTION IN RA NSAID PATIENTS



Showing mean IgG production with and without H_2O_2 (with and without 1:200 PWM) in $n = 4$ individuals; SEM were omitted for clarity.

groups on their cells than the other groups (Fig. 4.22).

results

In the light of the above, showing the thiol dependence of immunoglobulin synthesis and defective PWM stimulated immunoglobulin synthesis in RA NSAID (probably due to thiol oxidation on monocytes and lymphocytes [1]), and the raised spontaneous IgG and IgM synthesis causing hyperglobulinaemia, we turned to suppressor cell assays to see whether the inability to switch off immunoglobulin synthesis in RA NSAID patients is due to defective suppressor cell systems, which could themselves be thiol dependent.

4.8. SETTING UP THE CON-A INDUCED SUPPRESSOR CELL ASSAY FOR SUPPRESSION OF Ig SYNTHESIS.

The method is a modified version of that described by Dr. Georgina Smith [9]. Optimal parameters for both the CON-A suppressor cell generating system and co-culture indicator system were determined in normal controls as follows.

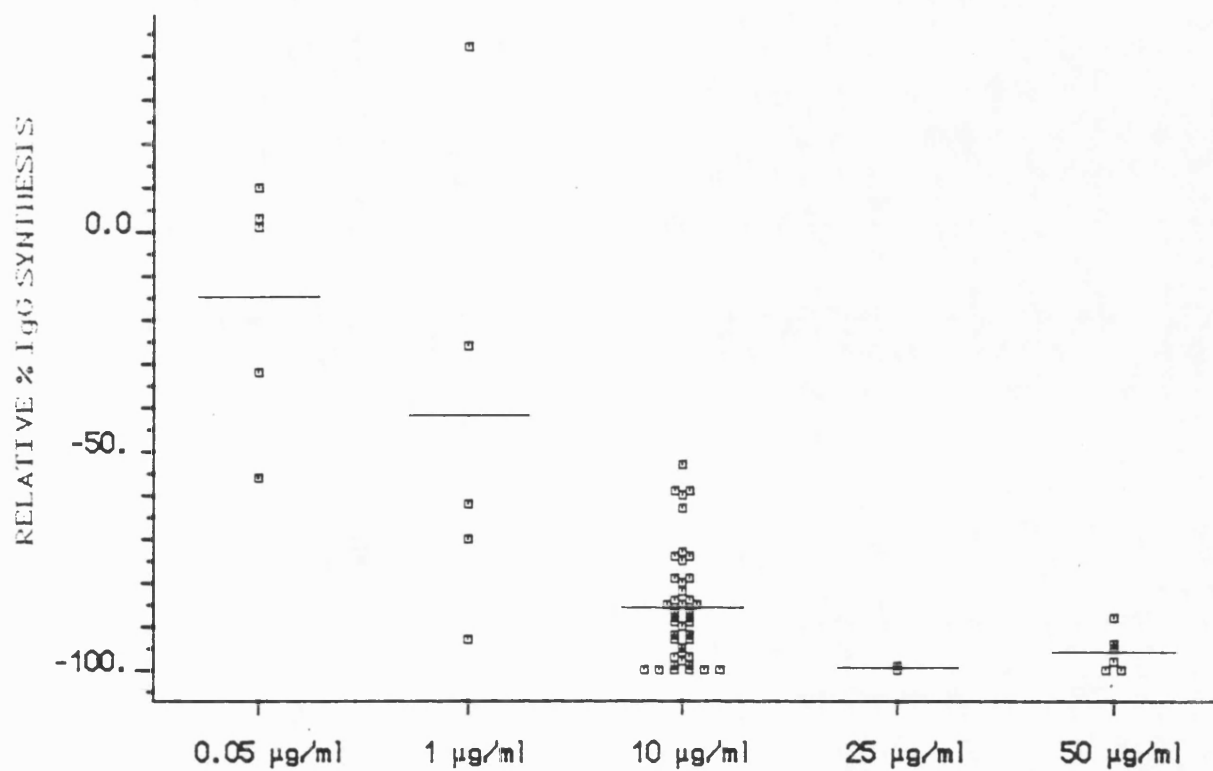
Initial experiments were set up to find the optimal dose of Concanavalin-A to use in the CON-A induced suppressor cell assay for IgG and IgM synthesis without appreciably affecting cell viability (Figs. 4.23-4.24). 10 μ g/ml CON-A fulfilled both requirements.

In the following paragraphs only parameters for the IgG assay are shown, but similar results were obtained for IgM.

IgG synthesis was found to be maximally suppressed at a PWM dilution of 1:200 (Table 4.10), which was previously found to be

FIG. 4.23

DOSE-RESPONSE CURVE FOR VARIOUS CON-A CONCENTRATIONS
ON % SUPPRESSION IgG SYNTHESIS IN NORMALS

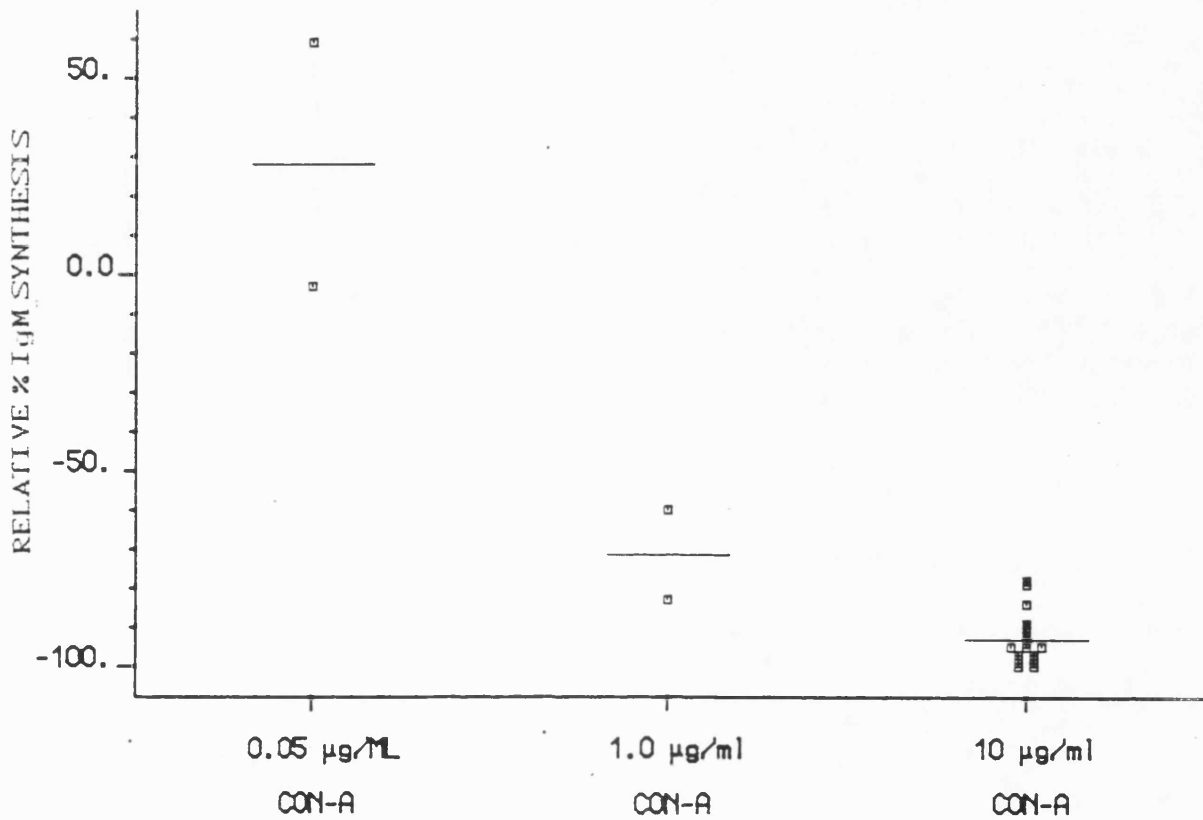


10 ug/ml Con-A represents an optimum concentration to use, giving high suppression of IgG synthesis and no loss in cell viability.

Horizontal bars represent median relative % IgG synthesis.

FIG. 4.24

CON-A DOSE-RESPONSE CURVE FOR CON-A INDUCED SUPPRESSOR
ACTIVITY ON IgM SYNTHESIS



10 ug/ml Con-A represents an optimum concentration to use, giving high suppression of IgM synthesis and no loss of cell viability.

Horizontal bars represent median relative % IgM synthesis.

TABLE 4.10

TABLE TO SHOW THAT % SUPPRESSION OF IgG SYNTHESIS IS MAXIMAL AT THE PWM DILUTION OF 1:200 WHICH WAS PREVIOUSLY FOUND TO BE OPTIMAL FOR STIMULATION OF IgG SYNTHESIS

PWM dil.	1/20	1/40	<u>1/200</u>	1/400	1/1000	1/2000
n	15	8	42	7	11	13
RELATIVE % IgG SYNTHESIS ± SEM	-80% ±8	-55% ±14	-86% ±2	-77% ±5	-70% ±7	-83% ±7

TABLE 4.11

REPRESENTATIVE SAMPLE OF ACTUAL DATA (ng/ 10^6 RESPONDER CELLS) FROM 16 NORMAL SUBJECTS OVER A RANGE OF PWM DILUTIONS SHOWING MAXIMUM % SUPPRESSION OF IgG SYNTHESIS AT 1/200 PWM DILUTION; RESULTS EXPRESSED AS MEAN ± SEM..

PWM DILUTIONS	1/40	1/200	1/400	1/1000	1/2000
UNTREATED COCULTURE - BACKGROUND	1041±439	1600±342	1493±590	1185±318	1340±523
CON-A COCULTURE - BACKGROUND	145±55	227±63	272±163	707±335	355±12
MEAN RELATIVE % OF IgG SYNTHESIS	-86	-86	- 82	-72	- 74

optimal for stimulating IgG synthesis (Table 4.2) Table 4.11 illustrates representative examples of actual IgG data used to calculate relative percent IgG synthesis values.

Table 4.12 shows that 3 mM and 0.3 M α -methylmannoside were generally found to be equally effective at removing CON-A from cell surfaces and preventing "carry over" to the responder cells in the coculture, but since 0.3 M was the more commonly quoted value in the literature, it was used in subsequent experiments. 0.3 M α -methylmannoside caused no significant decrease in cell yield, cell viability or subsequent response of cells to PWM. There was found to be no significant difference in the relative percent IgG synthesis whether the cocultures were set up for 8 days (in the exponential phase of IgG production (Fig. 4.12) or 12 days (around which maximal IgG production occurs (Fig. 4.12)); thus for convenience 8 days was chosen.

Table 4.13 shows that although cell viability fell gradually from the PBMNC separation stage onwards, it never fell below 90% even at the end of the 8 day PWM stimulated coculture period.

Table 4.14 shows that on comparison of three different methods for removing CON-A induced effector (putative suppressor cells) no significant difference was observed, but it was found to be more convenient to use a "rubber policeman" and this was also less prone to contamination problems.

Sample supernatants were collected for IgG analysis by carefully pipetting off a 150 μ l aliquot. Spinning the complete 200 μ l sample to

TABLE 4.12

INCREASING CO-CULTURE TIME DOES NOT ALTER RELATIVE % OF IgG SYNTHESIS OBSERVED; 2 DIFFERENT DOSES OF α -METHYL-MANNOSIDE WERE FOUND TO BE EQUALLY EFFECTIVE AT REMOVING CELL BOUND CON-A.

DAY OF HARVEST	3.0 mM α -M.M.	0.3 mM α -M.M.
DAY 8	- 81 % \pm 6 (n = 7)	- 87 % \pm 2 (n = 28)
DAY 12	- 87 % \pm 11 (n = 4)	- 86 % \pm 8 (n = 3)

Removal of Con-A was shown to be necessary since it otherwise caused simulation of responder cells, and therefore reduced suppressor activity.

TABLE 4.13

VIABILITY STUDIES AT DIFFERENT STAGES DURING THE CON-A INDUCED SUPPRESSOR ASSAY.

AFTER FICOLL SEPARATION	99 %	
AFTER 48 h WITH CON-A 10 μ g/ml	96 %	
AFTER 7 DAYS IN CO-CULTURE	- PWM	+ PWM
* UNTREATED CO-CULTURE	96 %	95 %
* CON-A CO-CULTURE	95 %	90 %
* UNT. CELLS HARVESTED, 2ME TREATED, WASHED, THEN COCULTURED	96 %	95 %
* CON-A TREATED CELLS HARVESTED, 2ME TREATED, WASHED, THEN COCULTURED	95 %	90 %

TABLE 4.14

DIFFERENT METHODS OF REMOVING EFFECTOR CELLS AFTER SUPPRESSOR
CELL GENERATION DOES NOT ALTER SUBSEQUENT RELATIVE % OF IgG SYNTHESIS.

SQUIRT/PIPETTE	SCRAPE WITH RUBBER POLICEMAN	GENTLY PIPETTE OFF NON ADHERENT CELLS
n = 13 - 83 % ± 5	n = 29 - 87 % ± 2	n = 2 - 93 % ± 6
N.S.		
N.S.		

TABLE 4.15

OPTIMAL TOTAL DENSITY FOR CO-CULTURE TO OBTAIN MAXIMUM
SUPPRESSION OF IgG SYNTHESIS WITHOUT INHIBITION OF CELL VIABILITY.

0.5×10^6 /ml	1.0×10^6 /ml	2.0×10^6 /ml/
n = 1	n = 6	n = 42
-83%	-79%±6	<u>-86%±2</u>

TABLE 4.16

REPRODUCIBILITY OF SUPPRESSOR CELL GENERATION AS SEEN BY ABILITY TO
SUPPRESS IgG SYNTHESIS ON DIFFERENT DATES.

SUBJ.	SEX	AGE	DATE	RELATIVE % IgG SYNTHESIS	DATE	RELATIVE % IgG SYNTHESIS	DATE	RELATIVE % IgG SYNTHESIS
1	F		21-04-86	-75	02-12-86	-80		
2	F		08-07-86	-100	01-12-86	-84		
3	F		23-06-86	-93	02-12-86	-95	12-02-86	-74
4	F		01-11-86	-100	10-03-87	-96		
5	F		01-12-86	-100	12-02-87	-85		
6	M		25-11-86	-74	10-03-87	-79		
7	M		02-12-86	-92	05-05-87	-87		
8	F		03-04-87	-79	07-03-87	-88		

remove cells was found to be not necessary as long as careful pipetting, so not to disturb the cells, was carried out.

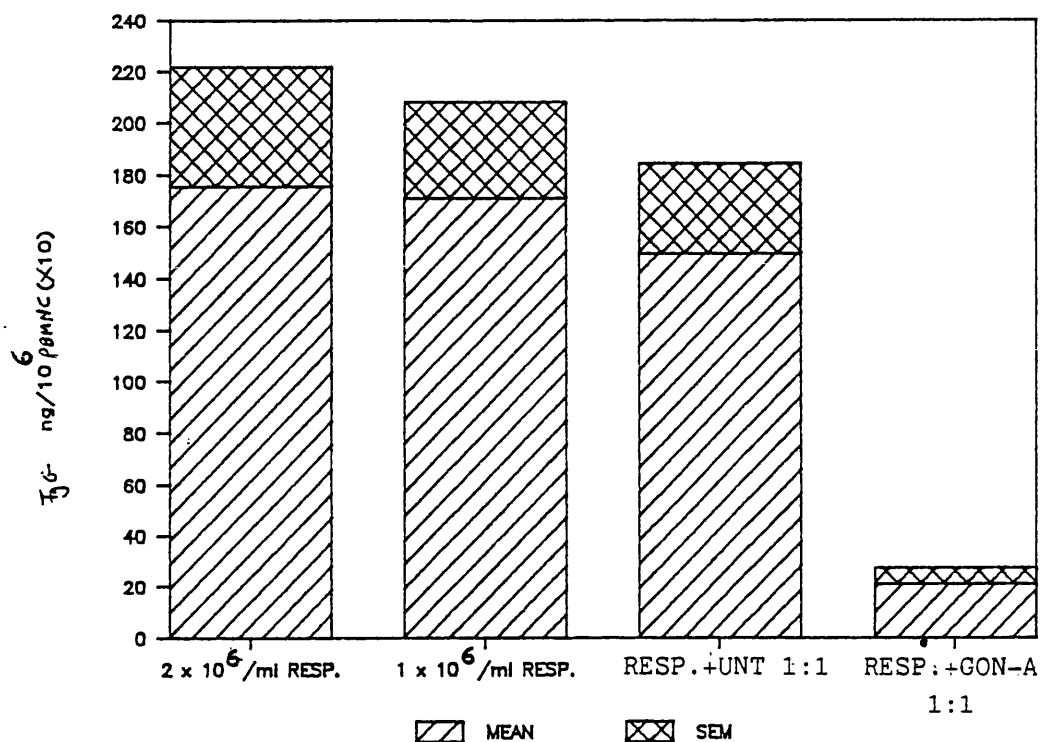
Table 4.15 shows that the optimal cell density for the coculture was 2×10^6 /ml without inhibiting cell viability.

Table 4.16 shows that the assay was highly reproducible within an individual over an extended period of time.

Fig. 4.25 shows that the only population of cells in the coculture able to produce IgG were the responder cells, the untreated or CON-A treated effector cells producing levels of IgG equivalent to background production. Likewise in all patient groups, including SLE, only responder cells made IgG, and PWM stimulated IgG production from effector cells, however treated, was always less than $200 \text{ ng} \times 10^6$ /ml, therefore like background levels. In 2 out of 23 RA NSAID patients effector cells produced up to $600 \text{ ng} \times 10^6$ /ml IgG.

It was found that only addition of CON-A treated effector cells, not untreated cells, to responder cells caused a significant reduction in IgG synthesis (Fig. 4.26). Fig. 4.26 shows the PWM stimulated IgG production in cocultures and from both 2×10^6 /ml and 1×10^6 /ml $200 \mu\text{l}$ cultures of responder cells. Both of the latter two were shown because it is difficult to decide which one is best to use for comparison with the UNT and CON-A treated cocultures, since the cocultures contain a final cell density of 1×10^6 /ml responder cells but a total cell density of 2×10^6 /ml PBMNC. Since the IgG output from UNT cocultures is greater than that from the 2×10^6 /ml responder cells culture alone, and it is already known that only responder cells

FIG. 4.25 EFFECT OF ADDITION OF UNTREATED OR CON-A TREATED CELLS
ON IgG SYNTHESIS FROM RESPONDER CELLS

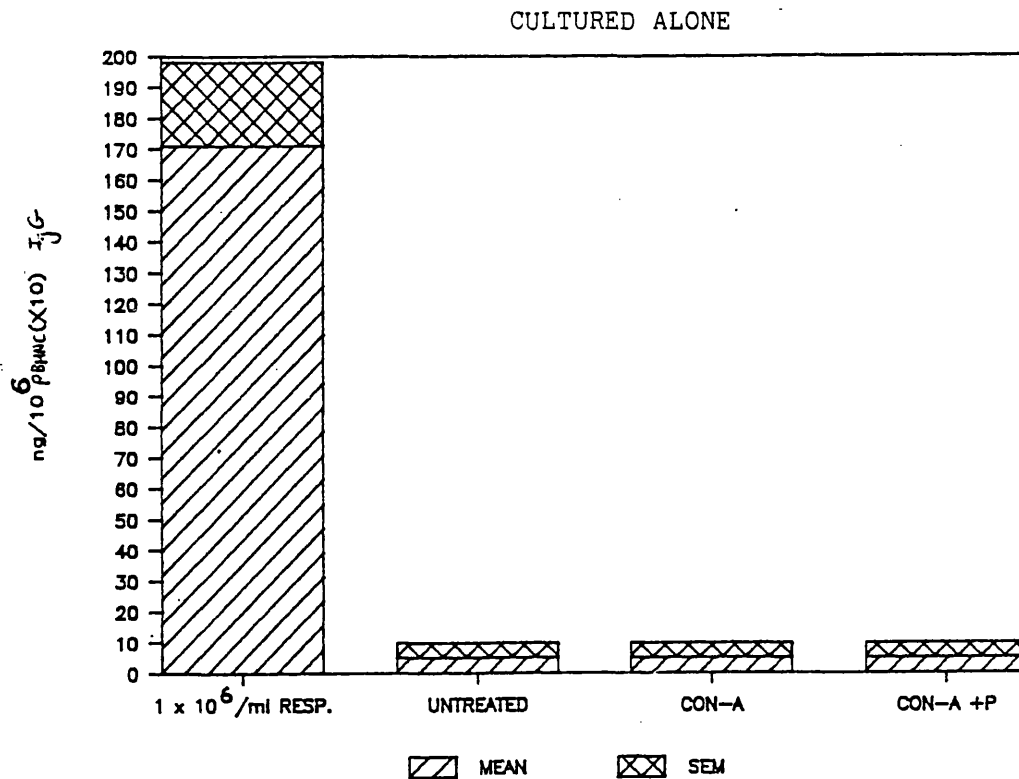


All cells were grown as 200 μ l cultures.

Only addition of 100 μ l of 2×10^6 /ml CON-A treated cells to 100 μ l of 2×10^6 /ml responder cells caused significant suppression of IgG synthesis from the responder cells.

Results represent mean IgG synthesis (1:200 PWM - BKG) \pm SEM for $n = 16$ normal subjects.

FIG. 4.26 IgG PRODUCTION FROM RESPONDER OR EFFECTOR CELLS



Effector cells were either untreated, treated with CON-A, or treated with CON-A then PHMPA (CON-A +P column). All cells were put up at 1×10^6 /ml and grown in 200 μ l cultures for 1 week. Results represent mean IgG synthesis (1:200 PWM - BKG) \pm SEM in $n = 16$ normal subjects.

are capable of making IgG, so in effect there are only 1×10^6 /ml IgG producing cells in the UNT coculture, it could be argued that the UNT cells have actually enhanced IgG output from the responder cells. This could be due to loss of short lived suppressor cells in the untreated effector cell population.

The optimum ratio of responder to effector cells was analysed in three ways:

one way involved varying the ratio of effector to responder cells in a total culture volume of 200 μ l, both cell populations originally at a cell density of 2×10^6 /ml (Table 4.17, part 1). This showed that addition of more CON-A-treated effector cells increased ^{the} percentage suppression of IgG synthesis, but could be criticized since one could argue that this was solely due to there being fewer responder cells present. However, the fact that IgG production for a given ratio of CON-A treated effector to responder cell gave significantly different results to the coculture of the untreated and responder cells at the same ratio, would indicate that it is a genuine effect attributable to the added CON-A-treated cells and not to the reduced proportion of responder cells. Reduced viabilities of the responder cells by chemicals released by the CON-A-treated cells could not account for the differences since viabilities were similar for all ratios. The method could be praised since total cell number and density were the same well to well and this is important since Table 4.2 clearly shows that cell density alone can influence IgG synthesis.

To overcome this criticism a second approach was taken, in which increasing numbers of CON-A treated effector cells were added to a

TABLE 4.17

TWO OTHER WAYS TO SHOW OPTIMAL RATIO OF EFFECTOR TO RESPONDER CELLS
FOR CON-A INDUCED SUPPRESSION OF IgG SYNTHESIS:

1) VARYING THE RATIO OF EFFECTOR:RESPONDER CELLS, KEEPING TOTAL
CULTURE VOLUME AT 200 μ l, AND BOTH CELL POPULATIONS AT 2×10^6 /ml.

RATIO REPRESENTS CELL VOLUMES IN μ l.

PERCENT VALUES REPRESENT THE MEAN RELATIVE PERCENT OF IgG SYNTHESIS IN
THREE NORMAL SUBJECTS.

50:150	67:133	100:100	133:67	150:50
-32%	-62%	-88%	-90%	-94%

2) VARYING THE NUMBER OF EFFECTOR CELLS ADDED TO A CONSTANT NUMBER
OF RESPONDER CELLS, AND BOTH CELL POPULATIONS AT 2×10^6 /ml.

RATIO REPRESENTS CELL VOLUMES IN μ l.

PERCENT VALUES REPRESENT THE MEAN RELATIVE PERCENT OF IgG SYNTHESIS IN
TWO NORMAL SUBJECTS.

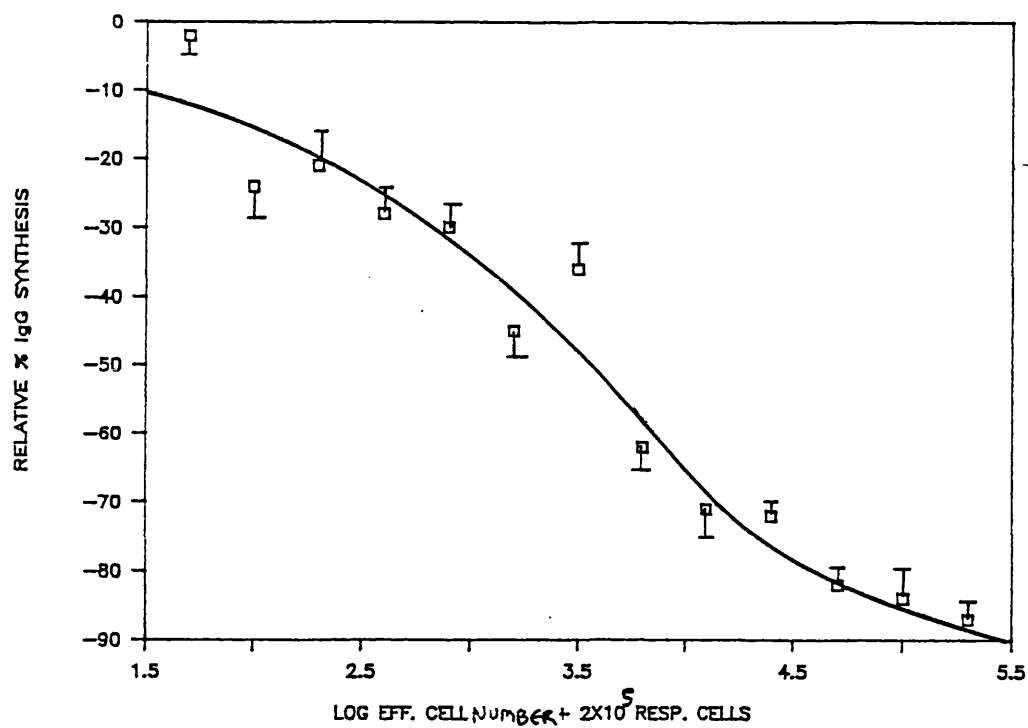
50:100	100:100	125:100	150:100	175:100	200:100	300:100
-50%	-80%	-78%	-76%	-75%	-68%	-67%

constant number of responder cells by varying the volume of the effector cells added. Both cell populations originally being at a cell density of 2×10^6 /ml. Table 4.17, part 2, shows that a one to one ratio gave maximum percentage suppression of IgG synthesis. However, one could argue that the wells were not comparable, since although total cell density remained the same well to well, total cell number did not, nor did individual densities of responder or effector cells. It could be praised in that the number of responder cells would be constant well to well.

A third approach was also adopted, where the CON-A induced effector cells were double diluted out in RPMI, each well containing 100 μ l RPMI + 10% FCS, and then a constant number of responder cells was added in 100 μ l volume. In this way no one could argue that suppression seen was due to reduction of responder cells added, since both their number and density were the same well to well, nor could it be argued that wells were not comparable due to different volumes well to well, since volumes were equivalent; however wells did vary in total cell number and density, so equivalent ratios of untreated to responder cells had to be set up simultaneously for comparable purposes. Fig. 4.27 clearly illustrates that reducing the number of CON-A induced effector cells reduces the percent suppression of IgG synthesis observed. A similar curve was obtained for IgM (data not shown).

Fig. 4.28 shows that CON-A increased the percentage of T_8 bearing cells in a 48 hours suppressor cell generating system.

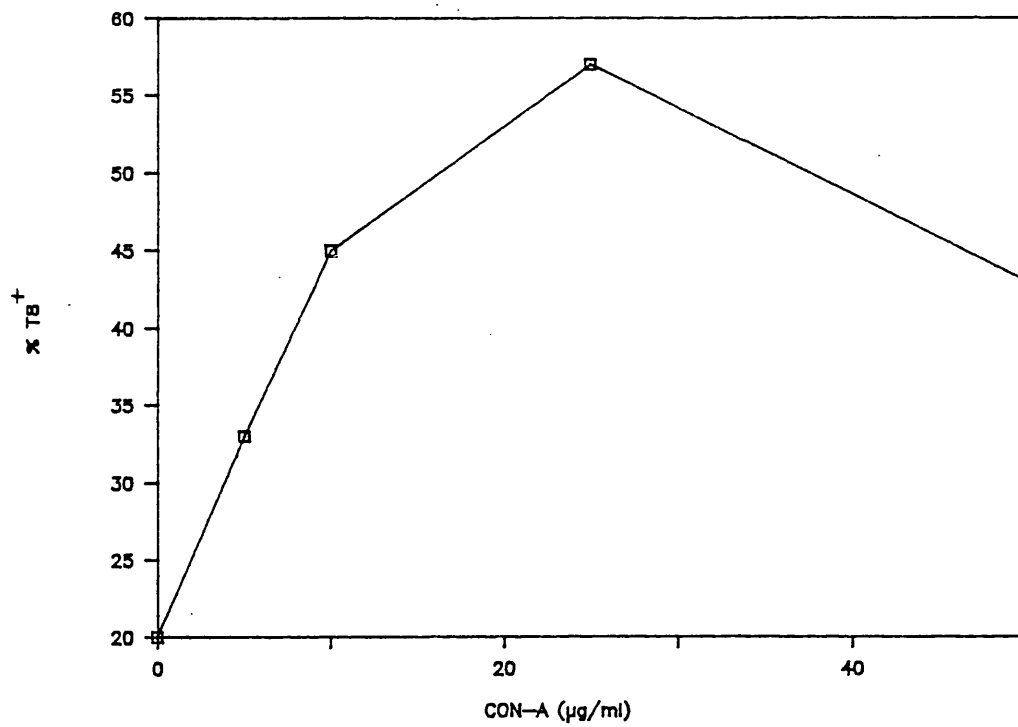
FIG.4.27 EFFECT OF DILUTION OF EFFECTOR CELLS TO A FIXED DENSITY
OF RESPONDER CELLS ON RELATIVE % IgG SYNTHESIS



Showing mean \pm SEM for n = 8 individuals.

FIG.4.28 EFFECT OF INCREASING DOSES OF CON-A ON % T8 +VE PBMNC

AFTER 48 HOUR CULTURE



Line represents mean of n = 2 observations.

Utilising a concentration of Royal Free Hospital anti-CD₈ and complement that should be able to lyse all CD₈⁺ cells in the system (Table 4.18), it was shown that removal of T₈ cells significantly reduced and in some cases totally abolished CON-A induced suppressor activity in the IgG (Table 4.19) and IgM assay systems (Fig. 4.29). Where there was not total obliteration of suppressor activity one must assume that other cells stimulated by the Concanavalin-A acted in a suppressive manner, such as the suppressor/inducer or "naive" subpopulation of CD₄⁺ cells and/or monocytic cells, but certainly one could say that the major cell acting suppressively was a CD₈⁺ lymphocyte.

4.9. COMPARISON OF CON-A INDUCED SUPPRESSOR CELL ACTIVITY IN VARIOUS PATIENT GROUPS AND NORMALS.

Utilising this method, CON-A induced suppression of IgG and IgM synthesis were investigated in various patient groups (tables 4.20-4.21 show actual data on which Figs. 4.30 and 4.31 are respectively based). Showing the actual data in this way, gives one an idea of the levels of Ig used to calculate relative % Ig synthesis, however it has no interpretable meaning since the concept of interest is what suppressor activity is like in each individual, and this can only be seen by calculating relative % suppression of IgG or IgM synthesis per individual, and comparing medians of these values between different patient groups as shown in Fig 4.30 and 4.31. However, Table 4.20 does serve to show that RA NSAID +RF had significantly raised IgG synthesis in their CON-A cocultures, but UNT

TABLE 4.18

TO SHOW THAT RFTB + C' CAUSE CELL LYSIS OF 100% OF T8⁺ CELLS PRESENT
IN THE CON-A INDUCED SUPPRESSOR CELL SAMPLE.

% T8 cells in CON-A induced suppressor cell population..... 45%
% T8 cells in CON-A induced suppressor cell population
after C' + RFTB treatment..... 0%

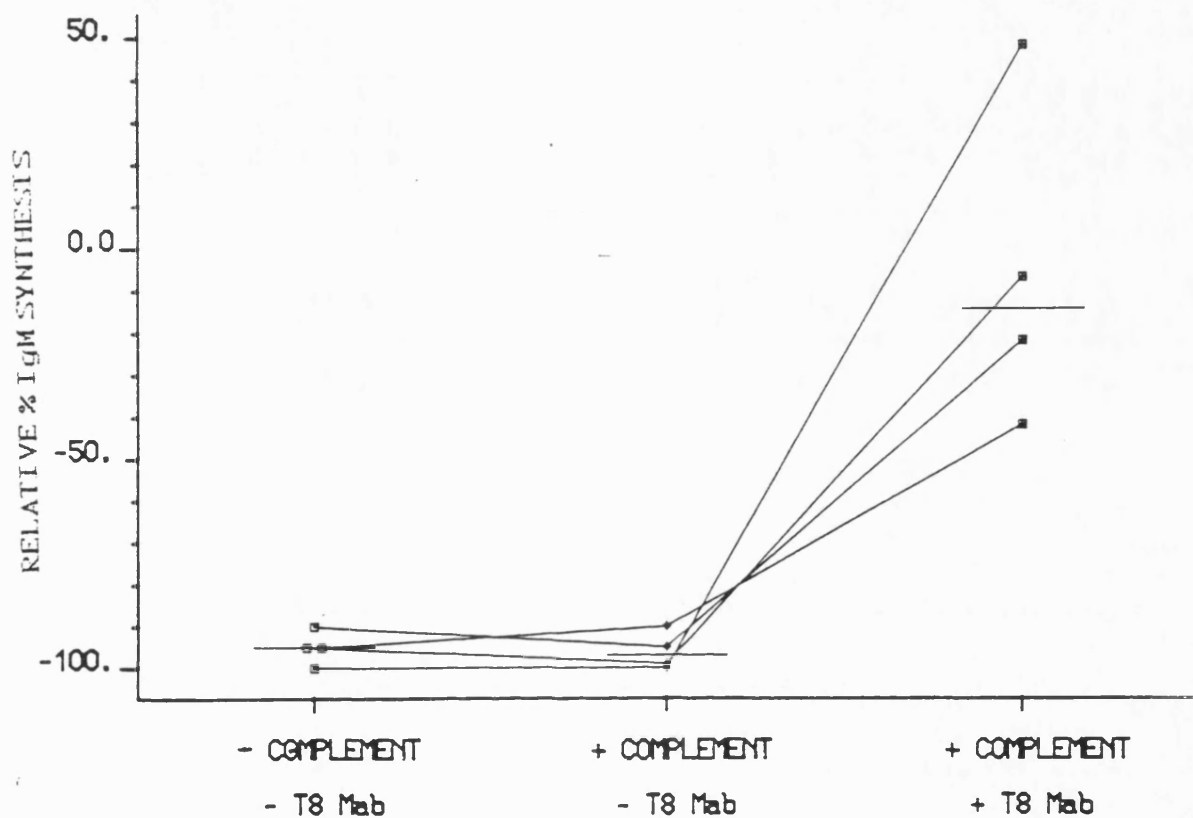
TABLE 4.19

INDIVIDUAL EXAMPLES TO SHOW EFFECT OF RFTB AND +C' ON CON-A INDUCED
SUPPRESSOR ACTIVITY ON IgG SYNTHESIS IN 3 NORMAL SUBJECTS.

Composition of coculture	IgG (ng/10 ⁶ responder cells) after 1/200 PWM - BKG	relative % IgG synthesis
PERSON A		
UNT-Coculture	668	-92%
CON-A Coculture	52	.
UNT + C' Coculture	667	-91%
CON-A + C' Coculture	62	
UNT + RFTB + C' Coculture	666	-38%
CON-A + RFTB + C' Coculture	203	
PERSON B		
UNT-Coculture	2103	-88%
CON-A Coculture	250	
UNT + C' Coculture	2102	-76%
CON-A + C' Coculture	778	
UNT + RFTB + C' Coculture	2105	5%
CON-A + RFTB + C' Coculture	2200	
PERSON C		
UNT-Coculture	2028	-85%
CON-A Coculture	313	
UNT + C' Coculture	2027	-88%
CON-A + C' Coculture	250	
UNT + RFTB + C' Coculture	2029	-33%
CON-A + RFTB + C' Coculture	1360	

FIG. 4.29

EFFECT OF REMOVAL OF T8 CELLS ON CON-A INDUCED
SUPPRESSOR CELL ACTIVITY FOR SUPPRESSION OF IgM SYNTHESIS



Complement has no effect on Con-A induced suppressor activity (+C' -T8 Mab vs. -C' -T8 Mab), but adding both complement and T8 Mab cause a significant reduction in induced suppressor activity (+C' +T8 Mab vs. +C' -T8 Mab).

Horizontal bars represent median relative % IgM synthesis.

TABLE 4.20

REPRESENTATIVE EXAMPLES OF ACTUAL CON-A INDUCED SUPPRESSOR DATA FOR
SUPPRESSION OF IgG SYNTHESIS (ng/10⁶ RESPONDER CELLS) AFTER BKG SUBTRACTION.

	NORMALS		RA+ NSAID		RA- NSAID		RA+/- NSAID	
COCULT.	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A
n =	42	42	19	19	4	4	23	23
MEDIAN	1121	126	1022	309	609	235	1022	336
RANGE	200	< 16	276	71	280	62	276	61
	8093	1006	3700	782	1420	1396	3700	1396
p vs. NORMALS	-	-	NS	< 0.05	*	*	NS	< 0.05
	RA+ D-PEN		RA- DPEN		RA+/- D-PEN		RA+/- D-PEN/AU	
COCULT.	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A
n =	5	5	3	3	8	8	10	10
MEDIAN	1688	353	1425	272	1547	347	1547	412
RANGE	901	221	1244	71	901	71	901	71
	2010	648	2002	910	2002	910	3365	910
p vs. NORMALS	*	*	*	*	NS	< 0.05	NS	< 0.01
			p vs. RA+/- NSAID		NS	NS	NS	NS
	AS PATIENTS		AS/PSA PATIENTS		SLE PATIENTS			
COCULT.	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A		
n =	12	12	14	14	4	4		
MEDIAN	1517	45	1517	45	1038	447		
RANGE	585	< 16	238	< 16	516	182		
	5280	750	5280	750	1468	573		
p vs. NORMALS	NS	NS	NS	NS	*	*		

* = NOT ENOUGH DATA FOR STATISTICAL ANALYSIS.

† = PRESENCE OF RF

- = ABSENCE OF RF

+/- = ALL PATIENTS IRRESPECTIVE OF RF STATUS

TABLE 4.21

ACTUAL CON-A INDUCED SUPPRESSOR DATA FOR SUPPRESSION OF IgM SYNTHESIS ($\text{ng}/10^5$ RESPONDER CELLS) AFTER BKG SUBTRACTION.

	NORMALS		RA+ NSAID		RA- NSAID		RA+/- NSAID	
COCULT.	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A
n =	20	20	11	11	4	4	15	15
MEDIAN	1028	55	322	170	119	86	258	110
RANGE	250	< 31	68	44	96	76	68	44
	7622	351	1980	1932	540	552	1980	1932
p vs. NORMALS	-	-	NS	< 0.05	*	*	< 0.05	< 0.005
	RA+ D-PEN/AU		RA- DPEN/AU		RA+/- D-PEN/AU		AS/PSA PATIENTS	
COCULT.	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A	UNT.	CON-A
n =	5	5	3	3	8	8	9	9
MEDIAN	426	52	588	79	507	76	1192	74
RANGE	104	< 31	242	76	104	< 31	52	< 31
	1100	106	1588	118	1588	118	1902	373
p vs. NORMALS	*	*	*	*	NS	< 0.01	NS	NS
			p vs. RA+/- NSAID		NS	NS	NS	NS
	SLE PATIENTS							
COCULT.	UNT.	CON-A						
n =	4	4						
MEDIAN	1961	952						
RANGE	70	110						
	3852	1794						
p vs. NORMALS	*	*						

* = NOT ENOUGH DATA FOR STATISTICAL ANALYSIS.

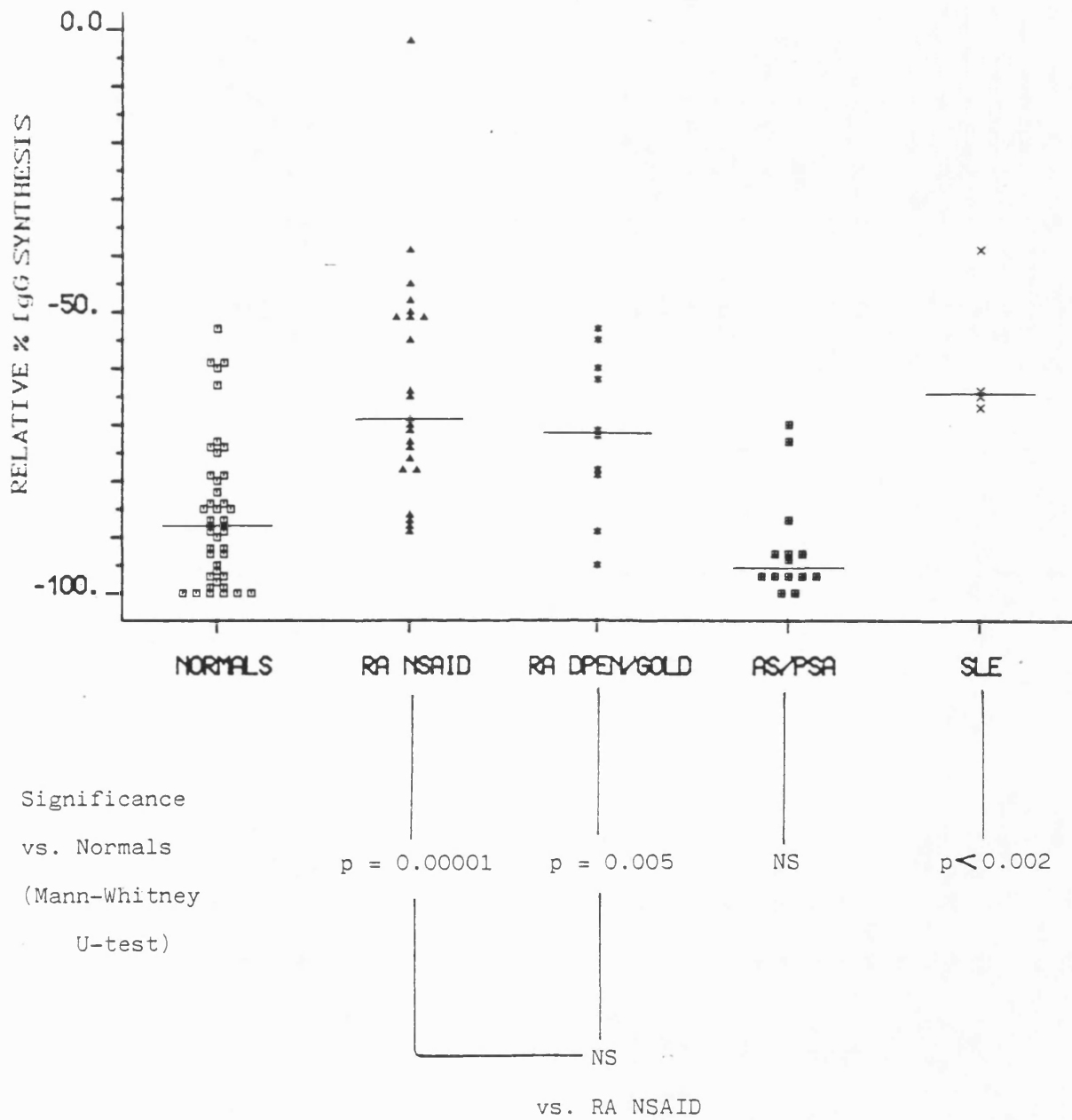
+ = PRESENCE OF RF

- = ABSENCE OF RF

+/- = ALL PATIENTS IRRESPECTIVE OF RF STATUS

FIG. 4.30

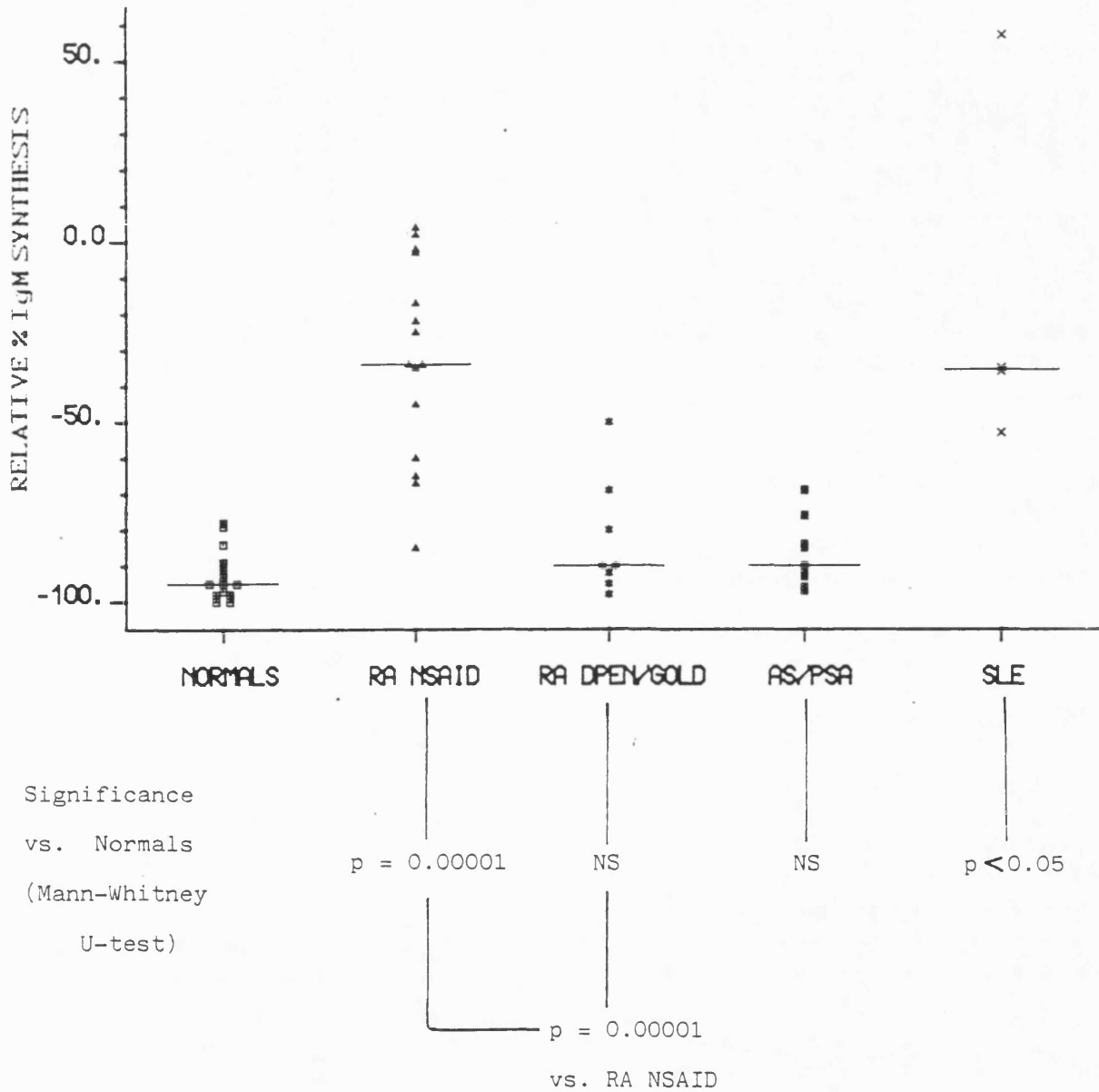
CON-A INDUCED SUPPRESSOR ACTIVITY ON IgG SYNTHESIS IN VARIOUS PATIENT GROUPS



Horizontal bars represent median relative % IgG synthesis.

FIG. 4.31

CON-A INDUCED SUPPRESSOR ACTIVITY ON IgM SYNTHESIS IN VARIOUS PATIENT GROUPS



Horizontal bars represent median relative % IgM synthesis.

cocultures were normal; RA NSAID -RF showed a tendency to reduced IgG synthesis in their UNT cocultures, and high synthesis in their CON-A cocultures, thus total RA NSAID +/- RF had normal IgG synthesis in their UNT cocultures but raised levels in their CON-A cocultures, resulting in an overall defective relative % suppression of IgG synthesis. RA D-PEN +/- RF and RA D-PEN/AU +/- RF had significantly raised IgG synthesis in their CON-A cocultures, but their UNT cocultures were normal, accounting for the defective suppressor activity shown in Fig. 4.30. AS and AS/PSA patients showed no defect in either coculture, and the 4 SLE patients showed raised IgG synthesis in their CON-A cocultures, accounting for their defective suppression of IgG synthesis. Table 4.21 shows RA NSAID +/- RF presented with significantly defective IgM synthesis in both UNT and CON-A cocultures, and since relative % IgM synthesis is calculated from a ratio of the latter to the former, a low IgM synthesis in the UNT cocultures probably accounts for the defect shown. The SLE patients only showed defective suppressor activity because of raised IgM synthesis in CON-A cocultures. The AS/PSA patients behaved similarly to normals in both the UNT and CON-A cocultures, but the RA D-PEN/AU patients showed significantly greater reduction in IgM synthesis in their CON-A cocultures than normals.

It can be seen from Fig. 4.30 that there was a significant reduction ($p = 0.00001$) in CON-A induced suppression of IgG synthesis in RA NSAID compared to normals, and surprisingly RA-DPEN/AU also showed defective CON-A induced suppressor activity ($p = 0.005$) compared to normals and there was no significant difference between RA

NSAID compared to RA-DPEN/AU. SLE patients were used as assay controls, known to show defective CON-A induced suppressor activity [8], and in agreement with the unanimous literature, significant defective CON-A induced suppression of IgG synthesis was observed ($p < 0.02$) compared to normals. AS/PSA patients, particularly those showing peripheral synovitis, were used as patient controls known to show chronic inflammation in synovial joints of similar histology to that in RA, they showed no CON-A induced suppressor cell activity defect for IgG synthesis, showing that CON-A induced suppressor activity defects are not common to all chronic inflammatory conditions but are more specific to certain rheumatic diseases, in particular SLE and RA.

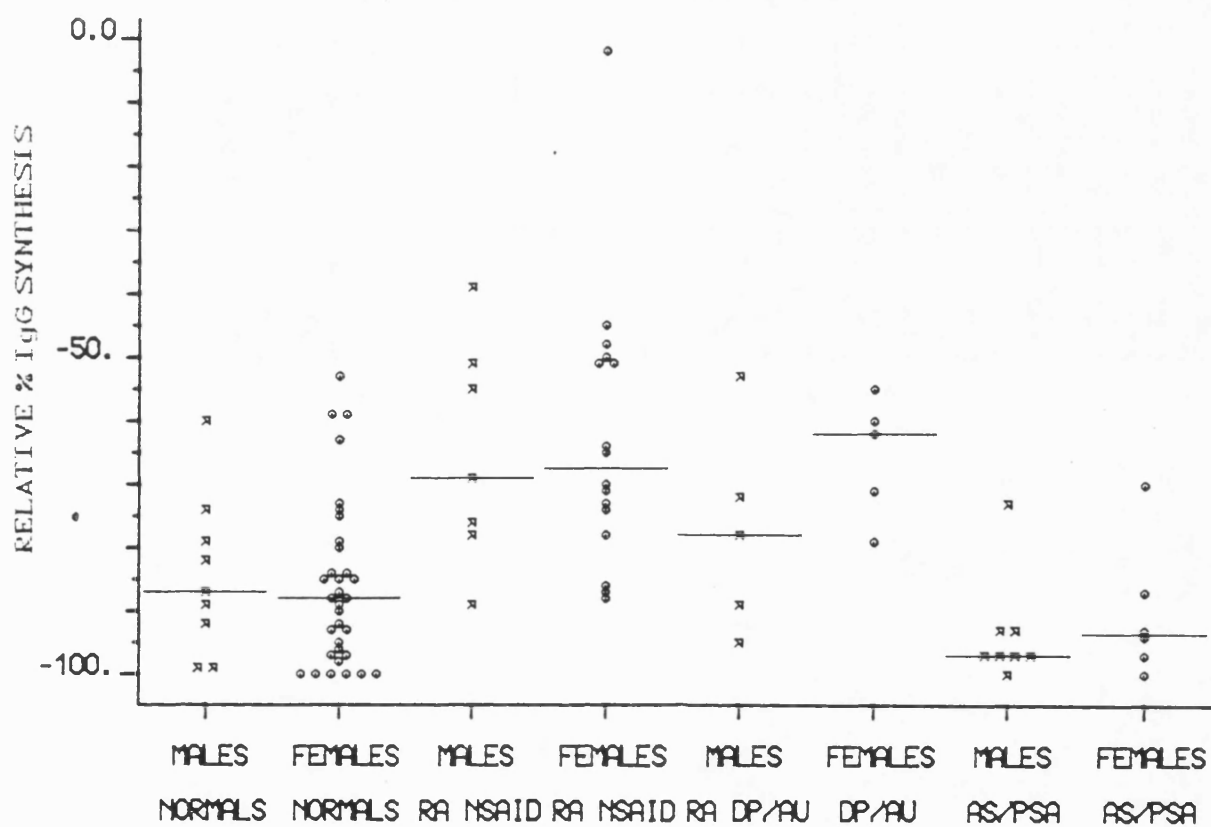
Fig. 4.31 shows that a similar trend was found for CON-A induced suppressor activity on IgM synthesis. RA NSAID showed a significant defect ($p = 0.00001$) compared to normals. However, there was no defect in the RA-DPEN/AU group, nor in the AS/PSA group of patients. Again the SLE patients were significantly defective compared to normals ($p < 0.05$). There was a significant difference between the RA NSAID and the DPEN group ($p = 0.00001$). In both the above figures +/- RF patients were grouped together since it was shown that suppressor activity did not correlate with the presence or absence of RF (see below).

No significant differences were observed between the sexes in any of the patient groups studied for CON-A induced suppression of IgG (Fig. 4.32) or IgM (Fig. 4.33) synthesis.

- In an attempt to correlate the defective suppressor activity observed in RA NSAID with relevant indices of disease activity (e.g.

FIG. 4.32

EFFECT OF SEX ON CON-A INDUCED SUPPRESSOR ACTIVITY ON IgG SYNTHESIS

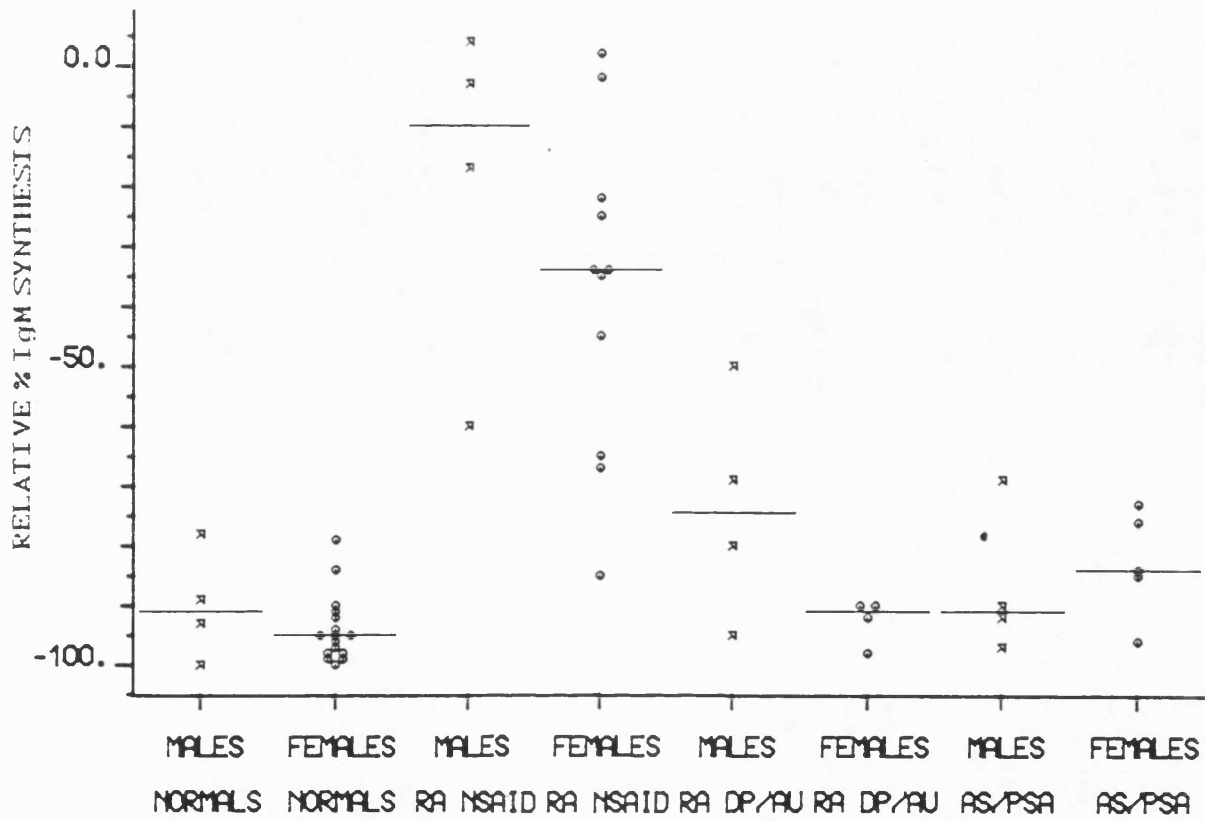


No significant differences within any group between sexes were observed using the Mann-Whitney U-test.

Horizontal bars represent median relative % IgG synthesis.

FIG. 4.33

EFFECT OF SEX ON CON-A INDUCED SUPPRESSOR ACTIVITY ON IgM SYNTHESIS



No significant differences within any group between sexes were observed using the Mann-Whitney U-test.

Horizontal bars represent median relative % IgM synthesis.

RF, EAD, EMS) or clinical indices of acute phase response (for example ESR, CRP, viscosity, WBC and PLTS), the following correlations were carried out.

There was no significant correlation and no evidence of a trend towards a correlation between CON-A induced suppressor activity for IgG ($n = 23$, $r = 0.14$) or IgM ($n = 15$, $r = 0.01$) synthesis and the presence of RF in RA NSAID, whose presence is usually regarded as indicative of more severe disease. So, in all future figures, RA NSAID patients with/without RF are grouped together; this has also been done for all other patient groups.

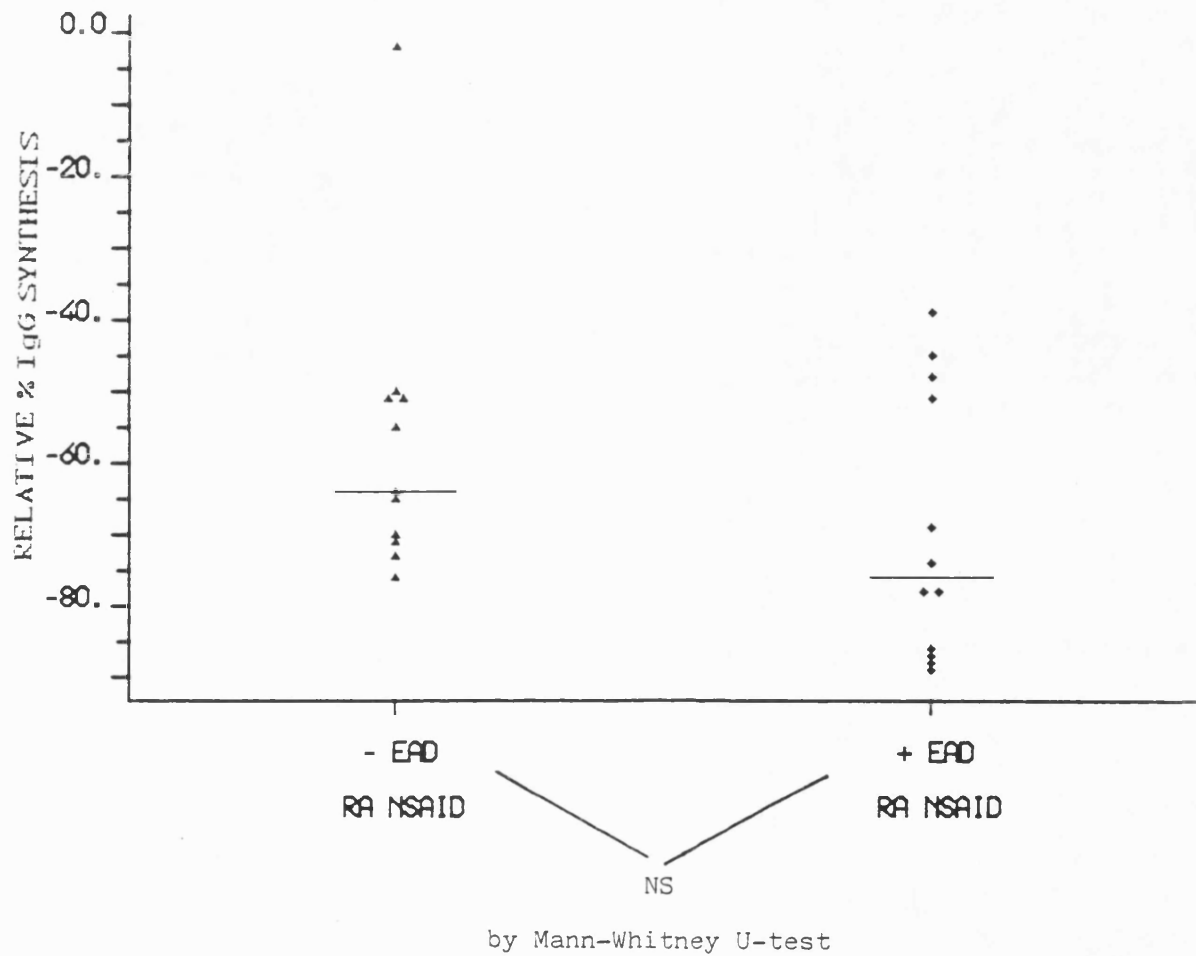
Figs. 4.34 and 4.35 show that RA NSAID patients presenting with a more severe active form of the disease, that is plus EAD, were equally defective in CON-A induced suppressor activity to those patients without EAD.

It was shown that a trend towards correlation existed, although not significant ($n = 7$, $r = 0.46$), between more defective suppressor activity for IgG synthesis and increased EMS, indicative of a more severe form of RA. There was insufficient IgM vs. EMS data to obtain a meaningful result.

Fig. 4.36 shows that there was a trend towards correlation, although not significant, in RA NSAID patients between CON-A induced suppression of IgG synthesis and ESR ($n = 6$, $r = 0.67$), and Fig. 4.37 shows a significant correlation ($n = 4$, $r = 0.87$, $p < 0.05$) between CON-A induced suppression of IgM synthesis and ESR, an increased ESR being associated with more active phases of the disease.

FIG. 4.34

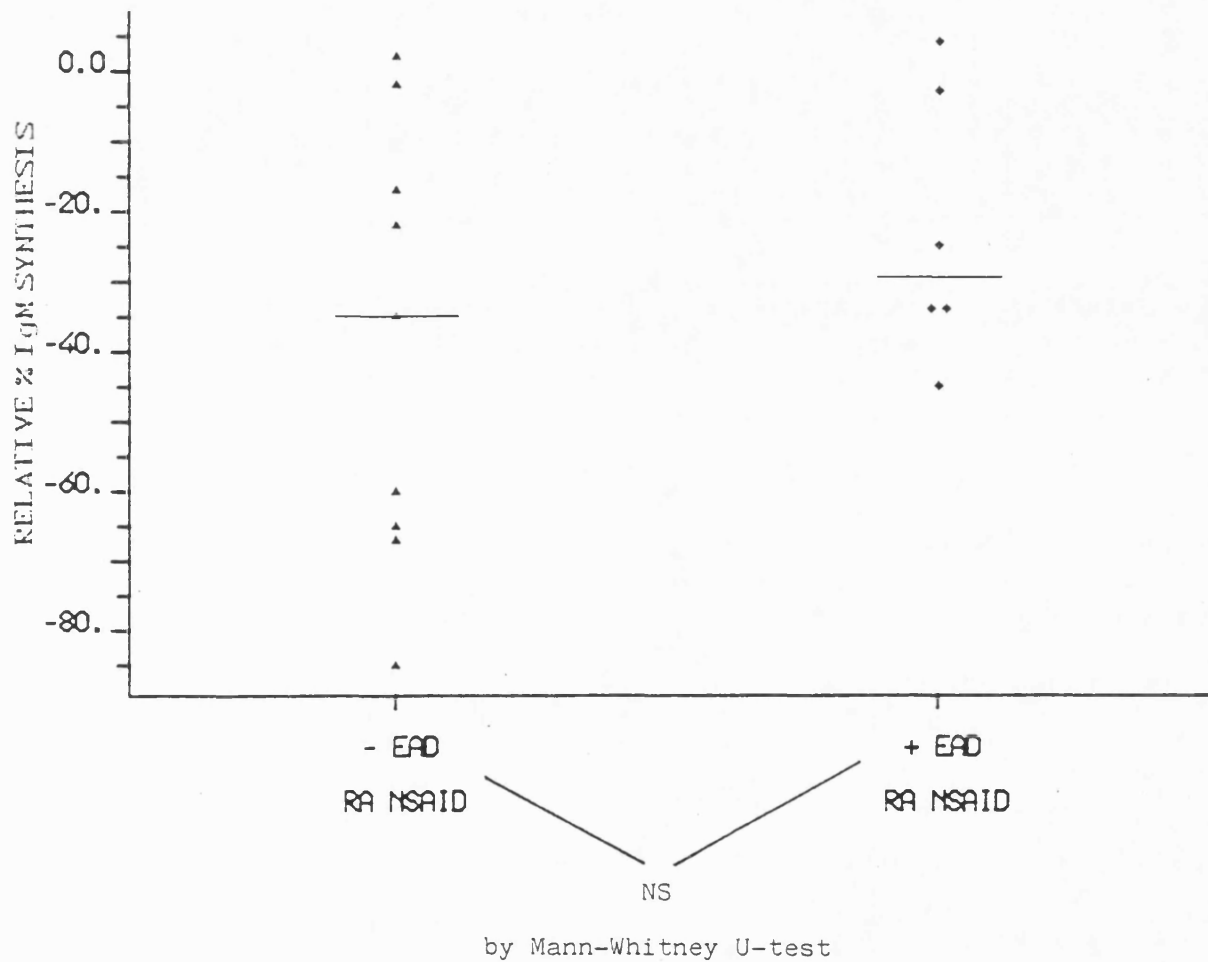
EFFECT OF EXTRA-ARTICULAR DISEASE ON CON-A INDUCED
SUPPRESSOR ACTIVITY ON IgG SYNTHESIS IN RA NSAID PATIENTS



Horizontal bars represent median relative % IgG synthesis.

FIG. 4.35

EFFECT OF EXTRA-ARTICULAR DISEASE ON CON-A INDUCED
SUPPRESSOR ACTIVITY ON IgM SYNTHESIS IN RA NSAID PATIENTS



Horizontal bars represent median relative % IgM synthesis.

FIG. 4.36 RELATIVE % IgG SYNTHESIS AND ESR IN RA NSAID (+/- RF)

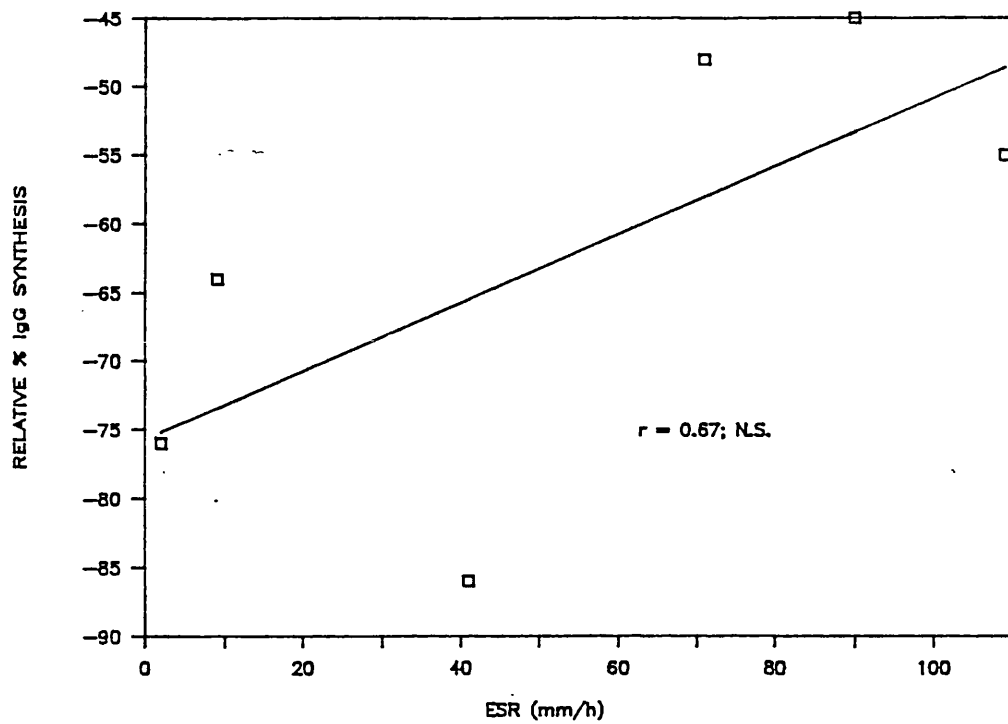
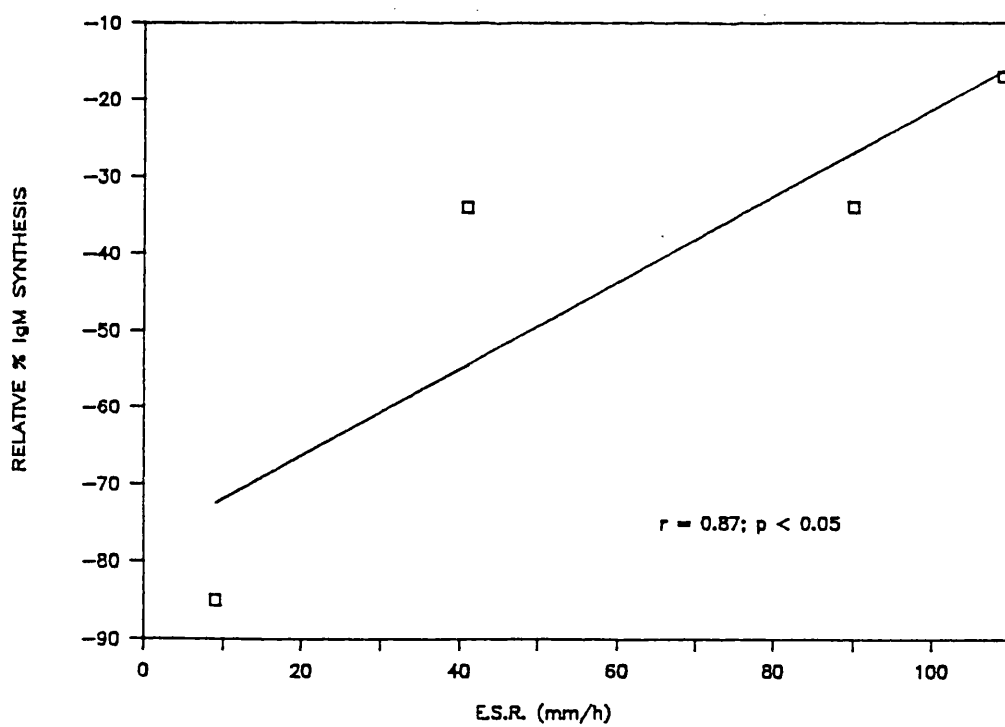


FIG. 4.37 RELATIVE % IgM SYNTHESIS AND ESR IN RA NSAID (+/- RF)



It was found that there was not even a trend towards a correlation between CON-A induced suppression of IgG synthesis and CRP ($n = 18$, $r = 0.12$) nor between suppression of IgM synthesis and CRP ($n = 12$, $r = 0.04$), which was thought to be unusual since a raised CRP is usually regarded as one of the best indicators of an acute phase response during a period of very active disease, since it rapidly rises and falls paralleling closely the inflammatory situation.

It was found that there was no significant correlation between CON-A induced suppression of IgG or IgM synthesis and viscosity ($n = 21$, $r = 0.11$; $n = 14$, $r = 0.29$ respectively), raised viscosity being associated with periods of more acute disease.

No significant correlation was found between CON-A induced suppression of IgG or IgM synthesis and WBC in RA NSAID ($n = 22$, $r = 0.18$; $n = 14$, $r = 0.28$ respectively), nor with PLTS ($n = 20$, $r = 0.08$; $n = 13$, $r = -0.27$ respectively), which is surprising since raised WBC or PLTS are usually associated with periods of active inflammation.

It was shown that there was no significant correlation between CON-A induced suppression of IgG or IgM synthesis and Hb levels ($n = 23$, $r = -0.22$; $n = 15$, $r = 0.02$ respectively), lowered Hb often being associated with very severe disease. It must however be remembered that patients with very low Hb levels (< 10 g/dl) were omitted from this project for ethical reasons, as stated in the method section, thus creating a bias for this correlation.

There was no significant correlation between CON-A induced

suppression of IgG or IgM synthesis and disease duration in RA NSAID ($n = 23$, $r = -0.38$; $n = 15$, $r = -0.38$ respectively), nor between CON-A induced suppression of IgG or IgM synthesis and age in RA NSAID ($n = 23$, $r = 0.11$; $n = 15$, $r = 0.00$ respectively).

It is not surprising that correlations were not found in all comparisons, since it should be remembered that rheumatoid arthritis (in common with other rheumatic diseases) varies tremendously in characteristics from patient to patient and almost represents a disease with unique characteristics per patient, so behaves as one of the least unified of diseases observed, e.g. one patient could present with reduced ESR and CRP and another patient present with a raised CRP and ESR and the former be classified on clinical grounds as having "active disease" and the latter not, despite the serological parameters indicating possibly the opposite picture. Therefore, it would possibly have been better not to correlate just two parameters but to use multivariate analysis to see whether there were associations between multiple parameters using a mainframe computer, however this facility was not available.

It was wondered whether those RA patients on D-PEN/AU treatment showing defective suppressor activity for IgG synthesis

had not been on treatment long enough for pharmacologically effective serum concentrations of the drugs to have been achieved. However, no significant correlation was found between CON-A induced suppression of IgG or IgM synthesis and length of time on a thiol containing drug ($n = 10$, $r = 0.13$ and $n = 8$, $r = 0.08$ respectively). In fig. 4.30 only 2 out of the 10 PEN/GOLD patients were on DPEN for

less than 6 months: one for 4 and one for 5 months, the former showing poor suppressor activity of -55% and the latter mediocre suppressor activity of -72%.

If good suppressor activity is taken as greater than -75%, then the 3 patients showing good suppression of -89%, -78% and -95% were on DPEN for 8, 96 and 10 months respectively and one patient on GOLD showing good suppressor activity of -79% had been on treatment for 48 months.

3 patients showing mediocre suppressor activity of -60%, -62% and -71% had been on DPEN for 7, 9 and 8 months respectively and a fourth patient showing poor suppressor activity of -53% had been on gold for 37 months. Since a serial study had not been carried out on these individuals, we shall never know if this is an improvement compared to before treatment, which would seem a reasonable suggestion since patients are usually put on these second line drugs when NSAID therapy fails to help and there is very severe erosive active disease; furthermore, these patients showed at the time of this study only moderately active disease. It could be suggested that treatment needs to be altered in these four patients, since 6 months is usually regarded as adequate time for pharmacologically effective doses to be reached, or that a raised dose of drug is required with careful monitoring for any harmful side effects which might ensue. In these subjects doses of DPEN of 375 mg/day, 375 mg/day and 625 mg/day respectively had been taken, and the fourth patient had been on intramuscular injections of aurothiomalate of 20 mg every 6 weeks. An inadequate dose of drug could therefore not account for the defective

suppressor activity observed. Interestingly, all 3 RA-DPEN patients were positive for RF, which is usually indicative of more severe RA. One of these patients showing mediocre suppression of IgG synthesis of 62%, but good suppression of IgM synthesis of 90%, was a 48-year-old female with disease duration of 27 years, with a markedly raised RF (2084 IU/ml), raised platelet count ($534,000/\text{mm}^3$), raised CRP (0.027 g/l), moderately active synovitis and nodules; however, normal serum SH, WBC and lymphocyte count were present. The 2 patients in the D-PEN/AU group showing worst suppressor activity in the IgG assays were both RF negative, and one had mild and the other no active peripheral synovitis.

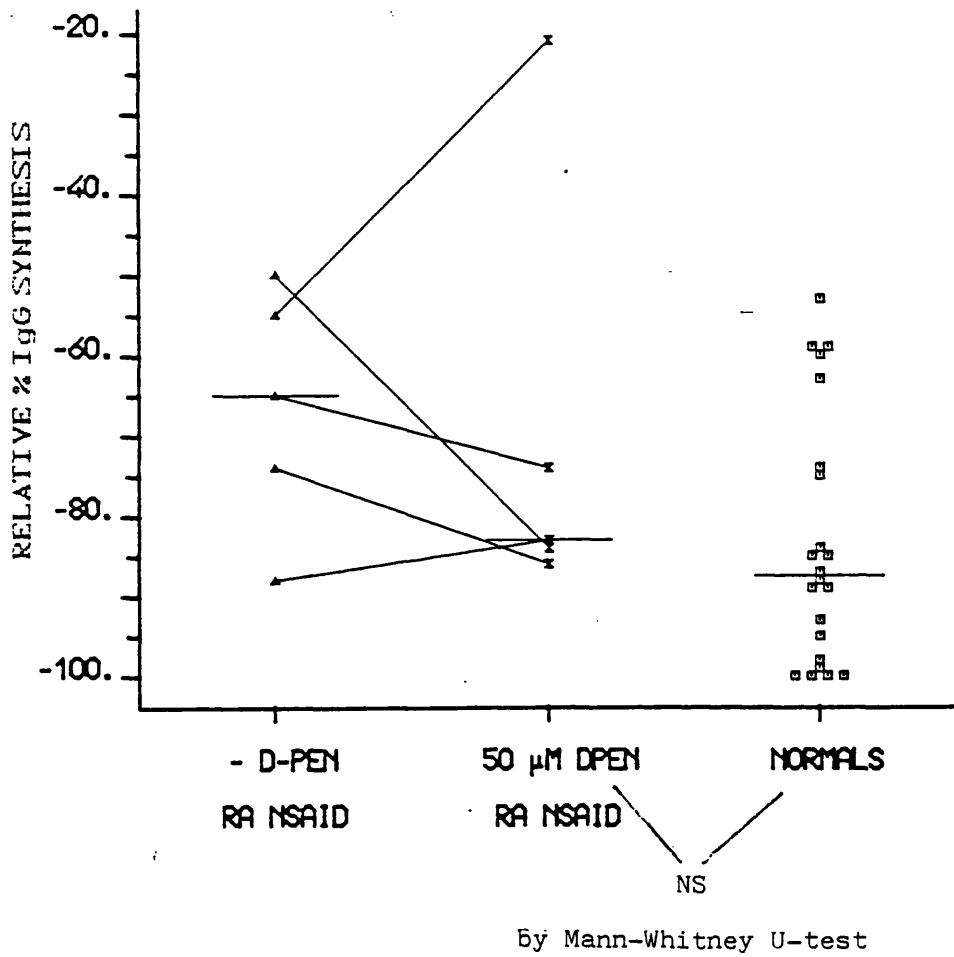
4.10. EFFECT OF D-PEN TREATMENT "IN VITRO" ON CON-A INDUCED SUPPRESSOR CELL ACTIVITY IN RA NSAID PATIENTS.

It was however observed that post-treatment of CON-A induced suppressor cells in RA NSAID patients with DPEN at $50\text{ }\mu\text{M}$ at $37\text{ }^{\circ}\text{C}$ for 1 hour (equivalent to a serum concentration that would be reasonable to expect after a 6 month course of DPEN) did normalise subsequent activity of suppressor cells in the IgG assay (Fig. 4.38); the same was true if $50\text{ }\mu\text{M}$ DPEN was left for 48 hours in the CON-A suppressor cell generating system (data not shown).

Similar improvement of CON-A induced suppressor activity for suppression of IgM synthesis was observed when DPEN was left in a CON-A suppressor cell generating system, however it did not normalise the data (figure 4.39), whereas fig. 4.31 showed DPEN/AU treated patients had normal CON-A induced suppressor activity for IgM

FIG. 4.38

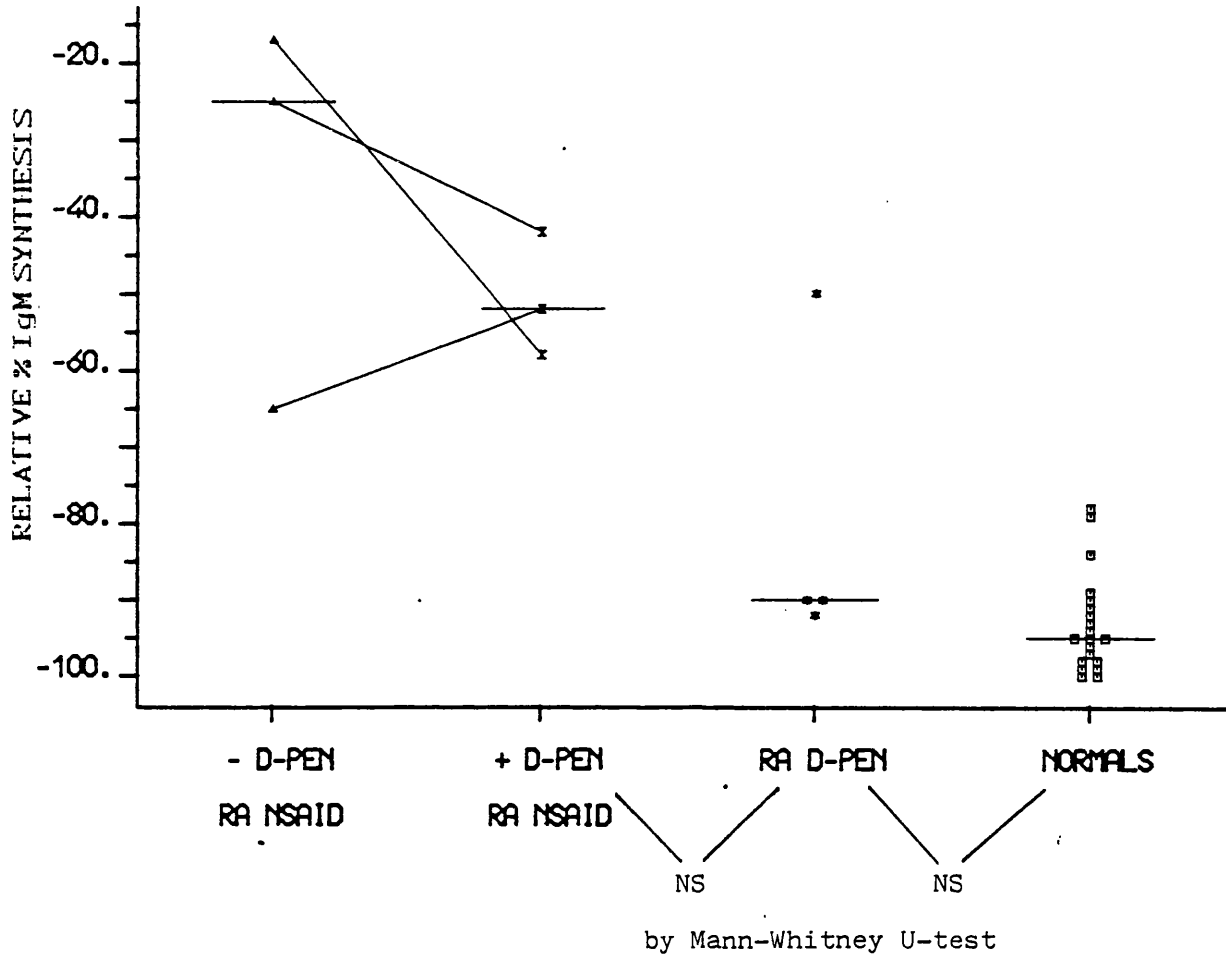
EFFECT OF POST-RX WITH D-PEN ON CON-A INDUCED SUPPRESSOR
ACTIVITY ON IgG SYNTHESIS IN RA NSAID PATIENTS



Horizontal bars represent median relative % IgG synthesis.

FIG. 4.39

EFFECT OF LEAVING D-PEN IN CON-A STIMULATED SUPPRESSOR CELL GENERATING SYSTEM ON SUBSEQUENT % SUPPRESSION IgM SYNTHESIS



Horizontal bars represent median relative % IgM synthesis.

synthesis.

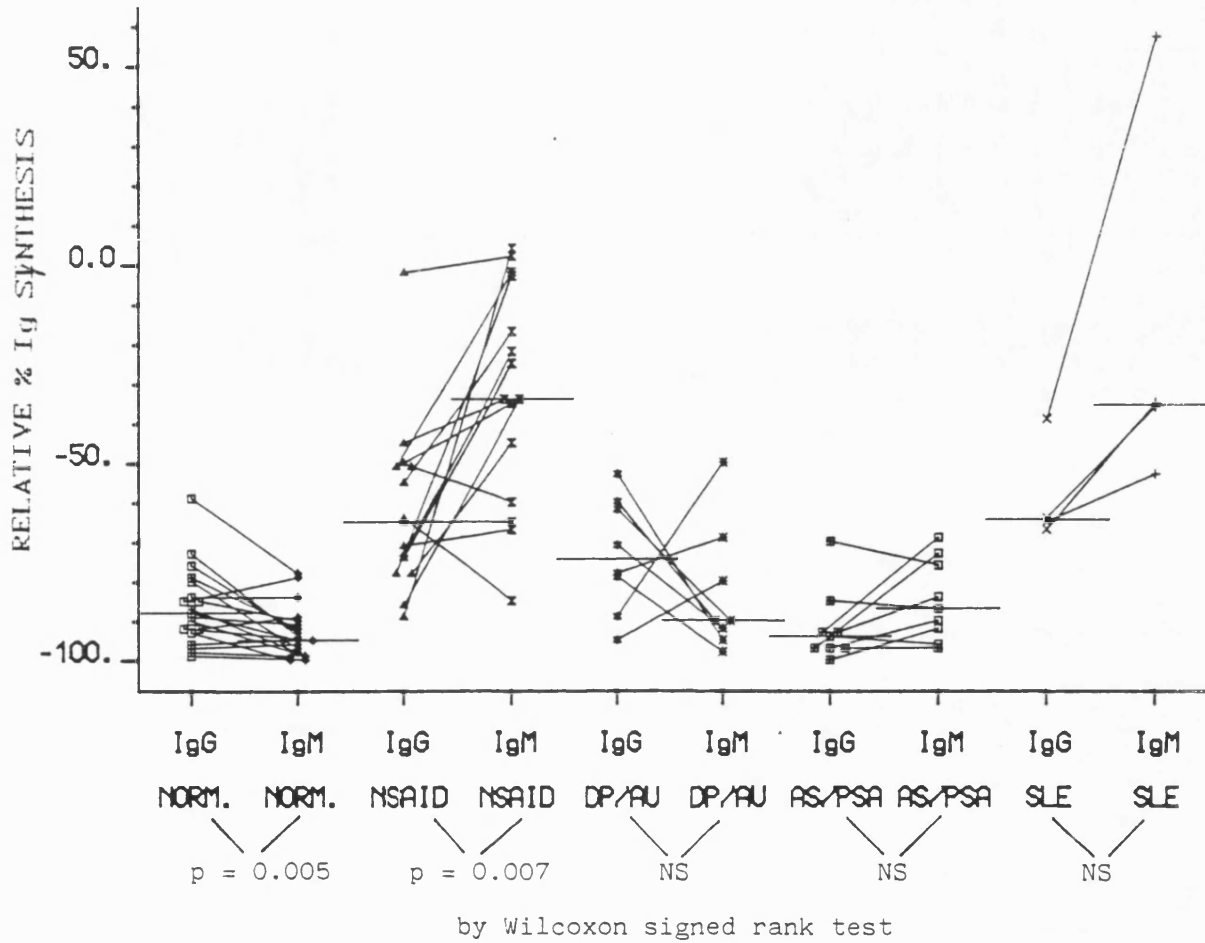
Comparison of CON-A induced suppression of IgG synthesis compared to IgM synthesis (Fig. 4.40) showed that there was a trend for increased suppression of IgM synthesis in normal subjects and a significantly more defective suppression of IgM synthesis compared to IgG synthesis in RA NSAID, however no correlation was found between relative % suppression of IgG vs. IgM synthesis ($n = 15$, $r = 0.15$) implying that different mechanisms are involved in IgG and IgM suppression, and significantly more defective suppression of IgG synthesis than IgM synthesis in RA-DPEN/AU patients. There was a trend for more suppression of IgG than IgM synthesis in AS/PSA and significantly more defective suppression of IgM synthesis than IgG synthesis in SLE.

This defective CON-A induced suppressor activity in RA NSAID and SLE patients for both IgG and IgM synthesis and the RA-DPEN/AU group for IgG synthesis was not due to differences in kinetics of expression of suppressor activity, as shown in Table 4.22, where it can be seen suppression steadily increased up to day 8 and was not increased by day 12 in all groups.

To see whether the defective suppressor activity in RA NSAID patients was exclusive to the CON-A induced suppressor system or common to other suppressor assays, the short-lived suppressor assay was also examined.

FIG. 4.40

CON-A INDUCED SUPPRESSOR ACTIVITY: COMPARISON FOR IgG AND IgM SYNTHESIS SUPPRESSION IN VARIOUS PATIENT GROUPS



Horizontal bars represent median relative % Ig synthesis.

TABLE 4.22

TABLE TO SHOW THE KINETICS OF THE CON-A INDUCED SUPPRESSOR CELL
ACTIVITY AS SHOWN BY ITS ABILITY TO SUPPRESS IgG SYNTHESIS FROM FRESH
RESPONDER CELLS

Patient group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 12
NORMALS n = 4	0%	-7%	-36%	-59%	-67%	-80%	-85%	-86%	-85%
RA-NSAID n = 3	0%	-13%	-28%	-29%	-36%		-55%	-74%	-75%
RA-DPEN n = 3	0%	-4%		-30%			-70%	-79%	-78%
AS/PSA n = 2/1	0%		-39%		-60%		-90%	-92%	-89%

Day 1 = day put up culture.

Day 8 = day supernatant harvested normally.

Percentages represent mean relative % suppression of IgG synthesis.

4.11. SETTING UP THE SHORT LIVED SUPPRESSOR CELL ASSAY

This method is a modification of that described by Dr Rui Victorino [7]. First an optimum concentration of CON-A for this system had to be found using normal individuals and was found to be 5 $\mu\text{g/ml}$, as shown in Fig. 4.41 (this is in fact a suboptimal dose for proliferation purposes as seen in Fig. 4.66).

Representative examples of CPM and how the suppressor index (SI), a formula used to describe suppressor activity in this system, were calculated, are shown in table 4.23.

4.12. COMPARISON OF S.I.

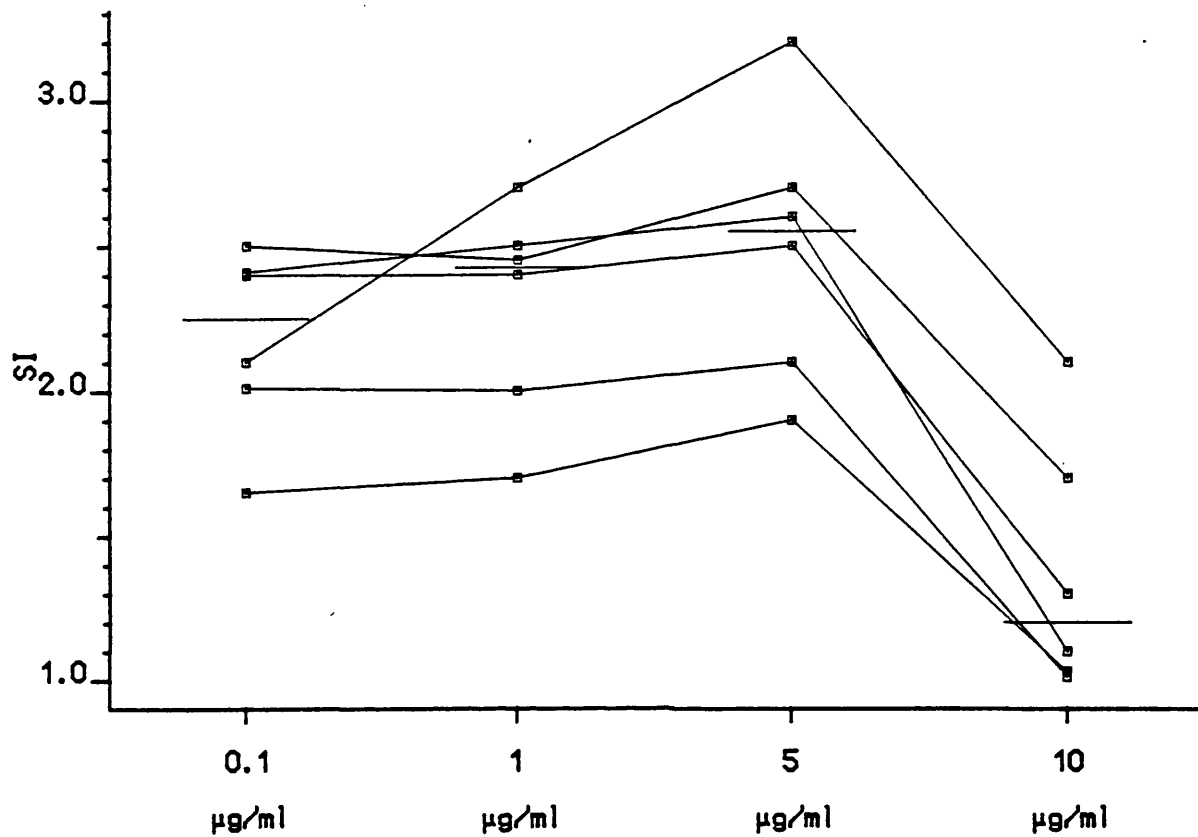
IN VARIOUS PATIENT GROUPS AND NORMALS.

It can be seen from fig. 4.42 that RA NSAID showed a significantly reduced SI, indicative of defective short-lived suppressor activity, compared to normals ($p < 0.01$) and compared to RA-DPEN ($p < 0.05$) and that RA-DPEN patients showed SI not significantly different to normals, a sign of normal short-lived suppressor activity. SLE patients also showed a significant reduction in SI compared to normals ($p < 0.01$).

This assay depends on the ability of cells to be stimulated by the mitogen CON-A, the SI being determined by a ratio whose denominator is CPM in response to CON-A. Many workers suggest RA NSAID patients show defective proliferation in response to mitogens, termed hyporesponsiveness [10-11] - said to be due to high spontaneous

FIG. 4.41

EFFECT OF VARYING STIMULATION DOSES OF CON-A
ON THE SHORT LIVED SUPPRESSOR ASSAY



5 ug/ml Con-A was found to be an optimum concentration to use
in the short lived suppressor assay.

Horizontal bars represent median suppressor index (SI).

TABLE 4.23

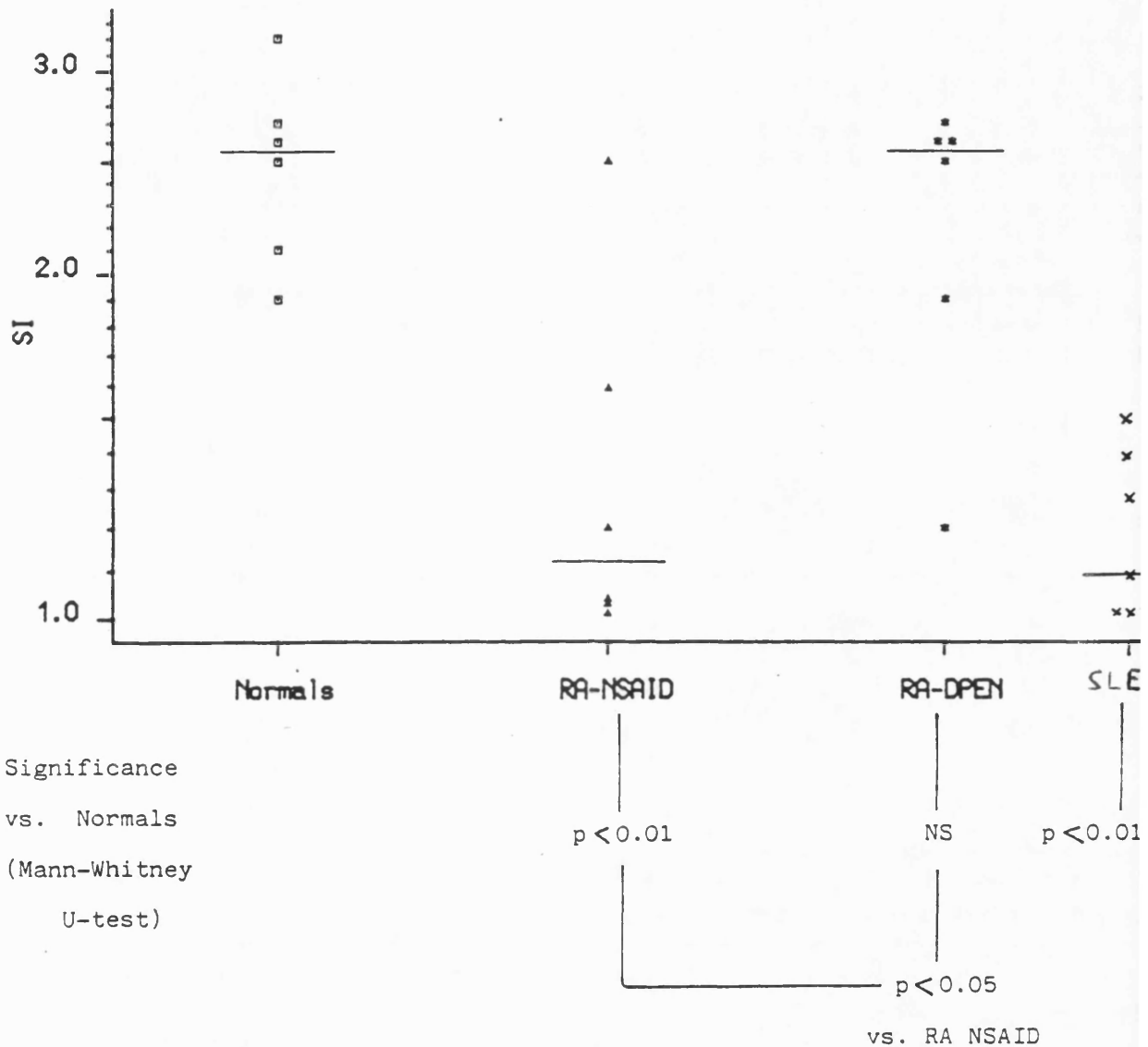
REPRESENTATIVE EXAMPLES OF SHORT LIVED SUPPRESSOR DATA

CON-A ($\mu\text{g/ml}$)	24 + 72 hr. Culture	96 hr. Culture	$\left[\frac{24 + 72}{96} \right] = \text{SI}$
NORMAL INDIVIDUAL A			
0.1	25225	10090	2.5
1	125661	51290	2.45
5 (opt. conc.)	272700	101000	2.7
10	204918	120540	1.7
RA-NSAID PATIENT B			
5	123040	102530	1.2
RA-DPEN PATIENT C			
5	261794	100690	2.6

[Values represent CPM - BKG counts, i.e. in absence of CON-A]

FIG. 4.42

COMPARISON OF SHORT LIVED SUPPRESSOR ASSAY SI IN
NORMALS, RA-NSAID, RA-DPEN, SLE



Horizontal bars represent median suppressor index (SI).

proliferation of PBMNC due to what has been suggested as prior activation of cells in vivo - it was therefore wondered whether defective short-lived suppressor activity data from RA NSAID was due to defective proliferation per se. Thus, a correlation between proliferation in response to CON-A at an optimum CON-A concentration (10 $\mu\text{g/ml}$) and SI were sought.

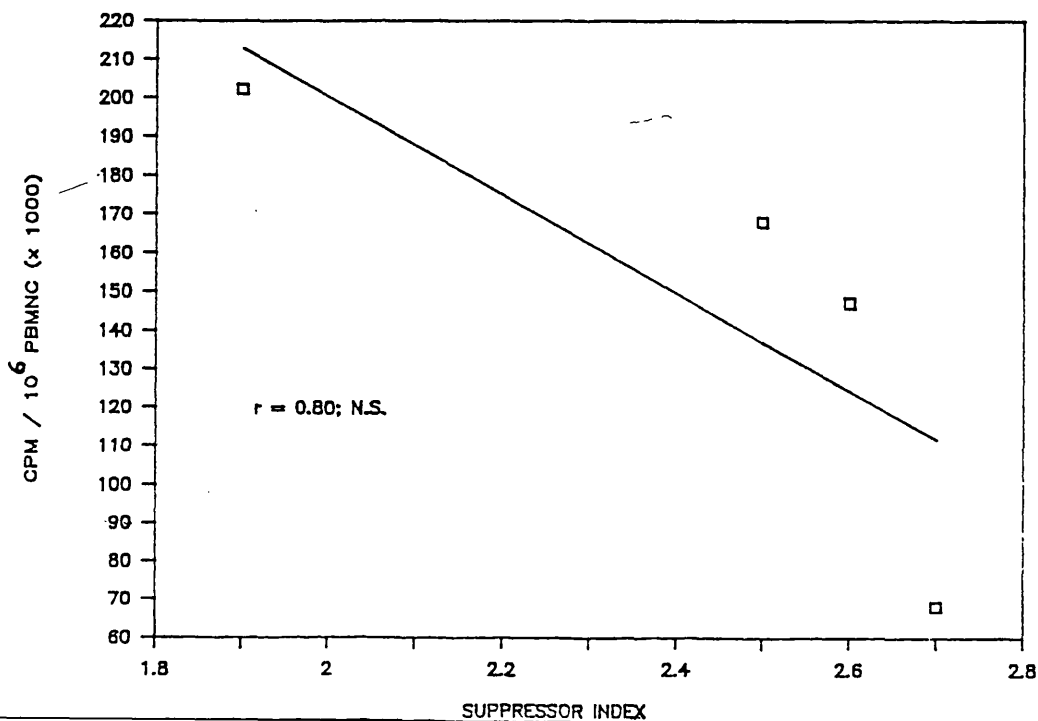
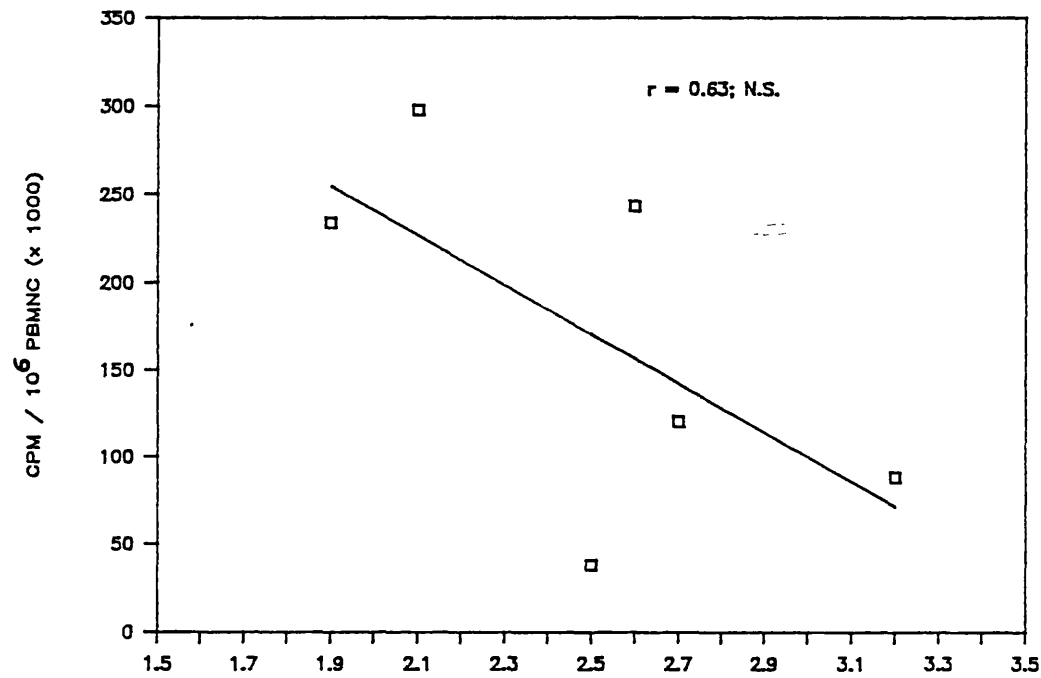
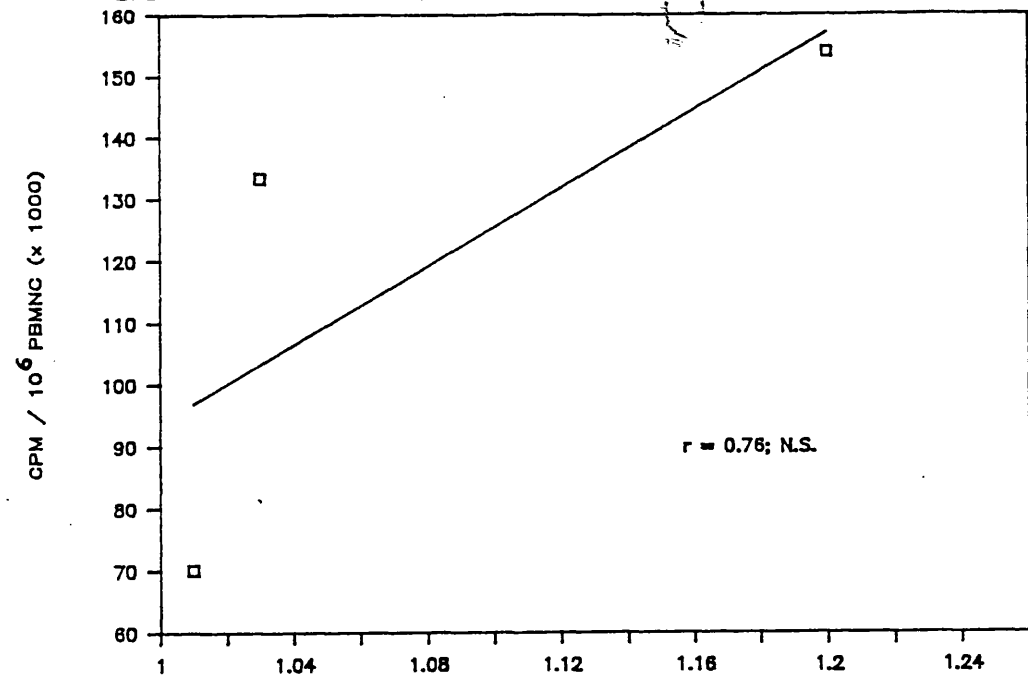
Fig. 4.43, part A, shows that there was a trend towards a correlation between proliferation and SI in RA NSAID, but it was not significant ($r = 0.76$), indicating that lower SI are achieved when cells are less able to proliferate in response to mitogen; however even in the RA NSAID patients showing a normal proliferative response the SI was still depressed. This cannot be the whole story, since it was found that there was a trend towards an inverse correlation between proliferation and SI (although not significant) in normals ($r = -0.63$, figs. 4.43, part B) and in RA-DPEN (Fig. 4.43, part C, $r = -0.8$), indicating that reduced proliferation responses in response to mitogen CON-A do not always lead to defective SI, in fact quite the opposite.

Comparing the CON-A induced suppressor activity for IgG synthesis and the short-lived suppressor activity across all normal and patient subjects, wherever parallel assays were carried out, a trend towards a negative correlation between the two assays, although not significant, was found ($n = 6$, $r = -0.38$), indicative that defective activity in one assay is also seen in the other.

The same trend, but more pronounced ($r = -0.87$, $n = 3$), towards a

FIGURE 4.23

CORRELATION OF PROLIFERATION IN RESPONSE TO 10 $\mu\text{g/ml}$ Con-A VS. SUPPRESSOR INDEX IN RA NSAID +/- RF (A), NORMALS (B), RA D-PEN +/- RF (C)



negative correlation, although not significant, was observed on comparison of the CON-A induced suppressor activity for IgM synthesis vs. the short-lived suppressor activity across all normals and patients, wherever parallel assays had been carried out.

Since it was wished to test the hypothesis that if defective suppressor activity was observed in RA NSAID, then it might be due to oxidation of functionally important thiol groups on the cell surface of cells responsible for this activity, it seemed logical to first approach the question by looking at the thiol dependence of CON-A induced suppressor induction and activity in normals and if either or both were found to be thiol sensitive; then, depending on the answer, efforts would be made to correct the defective CON-A suppressor activity in RA NSAID in vitro by adding the thiol reducing agent 2-ME before the CON-A suppressor cell generating system, or leaving it in a CON-A suppressor cell generating system, or treating cells with 2-ME after CON-A induced suppressor generation.

4.13. EFFECT OF PHMPA ON "INDUCTION" AND "ACTIVITY" OF CON-A INDUCED SUPPRESSOR CELL EXPRESSION IN NORMALS.

Table 4.24 shows that pretreatment of PBMNC with ammonium chloride before CON-A induced suppressor cell generation, to remove red cells whose surface thiol groups would otherwise react with added PHMPA and reduce its effective dose, did not reduce cell viability nor subsequent suppression of IgG synthesis. Figs. 4.44-4.45 show that pretreatment with 50 μ M and 100 μ M PHMPA resulted in no significant differences in CON-A induced suppressor activity for IgG or IgM

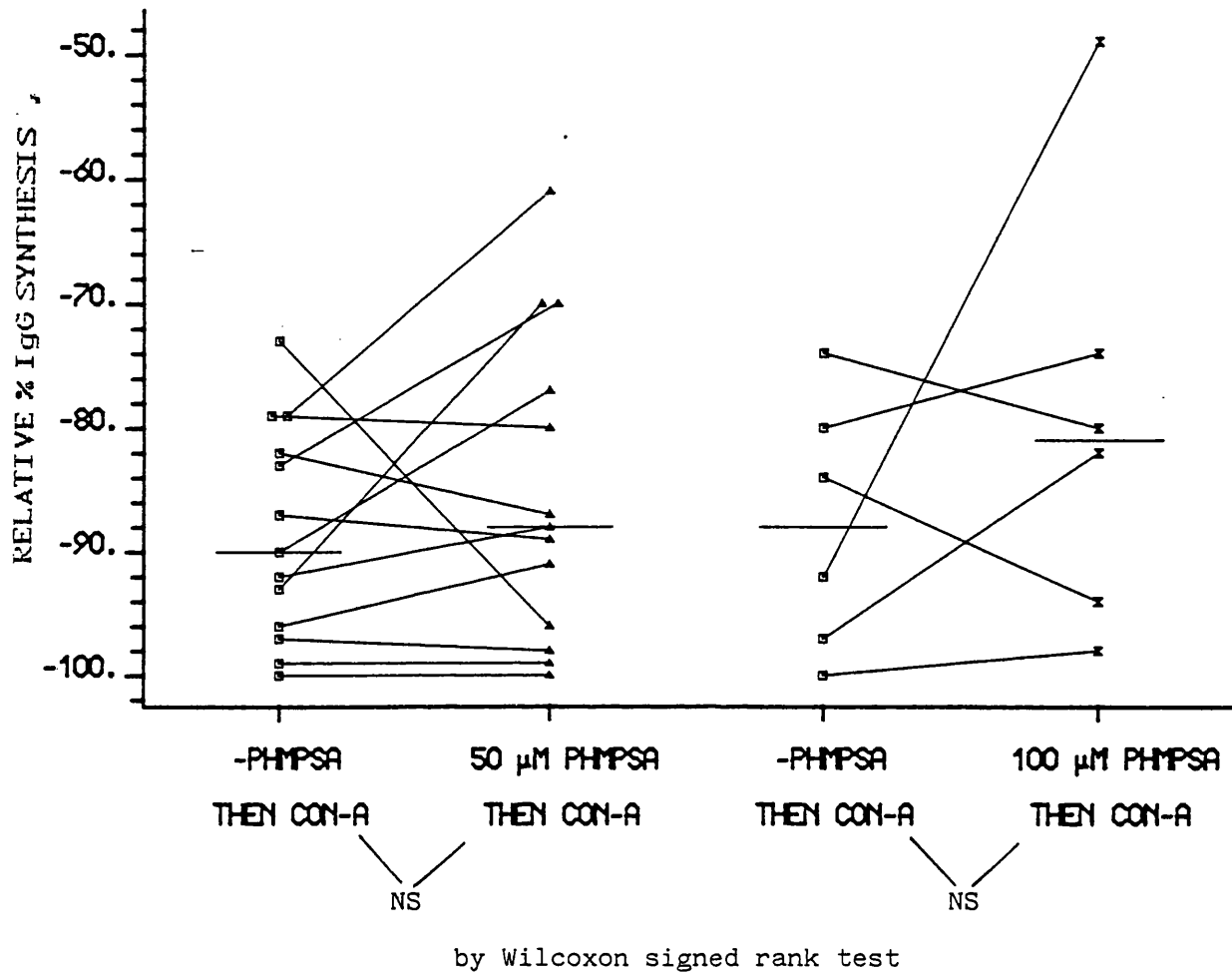
TABLE 4.24

PRE-TREATMENT OF PBMNC WITH NH_4Cl BEFORE CON-A INDUCED SUPPRESSOR CELL INDUCTION DOES NOT REDUCE CELL VIABILITY, NOR REDUCE RELATIVE % OF IgG SYNTHESIS BY COMPARISON WITH CELLS NOT TREATED WITH NH_4Cl .

	NH_4Cl TREATED CELLS	UNTREATED CELLS
	n = 20	n = 22
RELATIVE % IgG SYNTHESIS (MEAN \pm SEM)	-88 \pm 2%	-83 \pm 3%
CELL VIABILITY	95%	95%

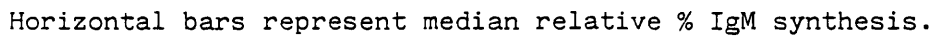
FIG. 4.44

EFFECT OF PRE-TREATMENT WITH PHMPA ON "INDUCTION"
OF CON-A INDUCED SUPPRESSOR ACTIVITY IN NORMALS



Horizontal bars represent median relative % IgG synthesis.

EFFECT OF PRE-TREATMENT WITH PHMPA ON 'INDUCTION' OF CON-A INDUCED SUPPRESSOR ACTIVITY ON IgM SYNTHESIS

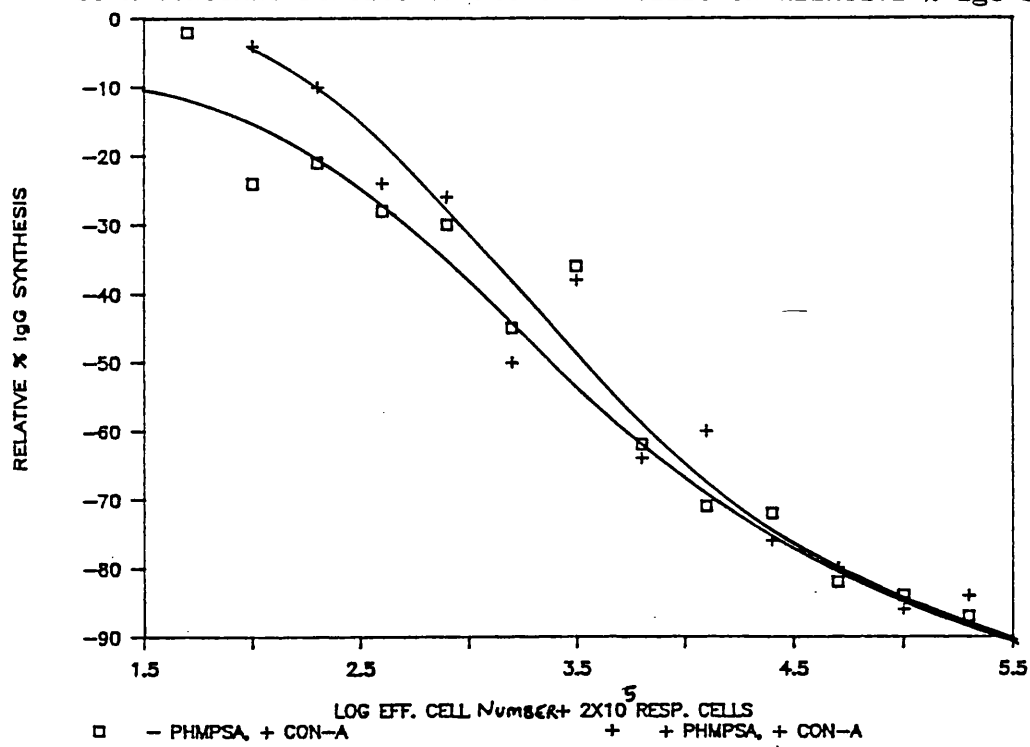


Horizontal bars represent median relative % IgM synthesis.

synthesis respectively, nor were significant differences found using suboptimal or superoptimal dilutions of PWM. This was confirmed in Fig. 4.46, which shows that when suppressor cells with/without PHMPSA pretreatment were diluted out with a constant density of responder cells, parallelism between the two curves of relative percent IgG synthesis was seen. The same was found in AS and RA-DPEN patients (data not shown). It should be noted of the 13 normals studied using 50 μ M PHMPSA 8 showed no significant differences in suppression of the IgG synthesis, but 4 showed some loss of suppressor activity, and one showed a significant gain in suppressor activity. Of the 10 normals studied using 50 μ M PHMPSA, 9 showed no significant differences in suppression of the IgM synthesis, but one showed a significant loss of suppressor activity.

However, figs. 4.47-4.48 show that once formed the activity of the CON-A induced suppressor cells are sensitive to thiol blockade in both IgG and IgM synthesis assays respectively. The same was found for AS and RA D-PEN patients (data not shown). Ammonium chloride treatment is not needed here, since red cells would have been lysed during the 48 hour preculture period. Fig. 4.47 shows that in the IgG assay post-treatment with 15 μ M PHMPSA had no significant effect, but 50 μ M showed significant reduction ($p < 0.01$) in suppressor activity and this effect was even more pronounced with 100 μ M PHMPSA. Similar trends were found in the IgM assay. In both assays, using 50 μ M PHMPSA, not all subjects were equally responsive to thiol blockade. In the IgG assay 3 out of 9 showed no significant effect, 2 out of 9 a little effect, and 4 out of 9 showed a dramatic inhibition of

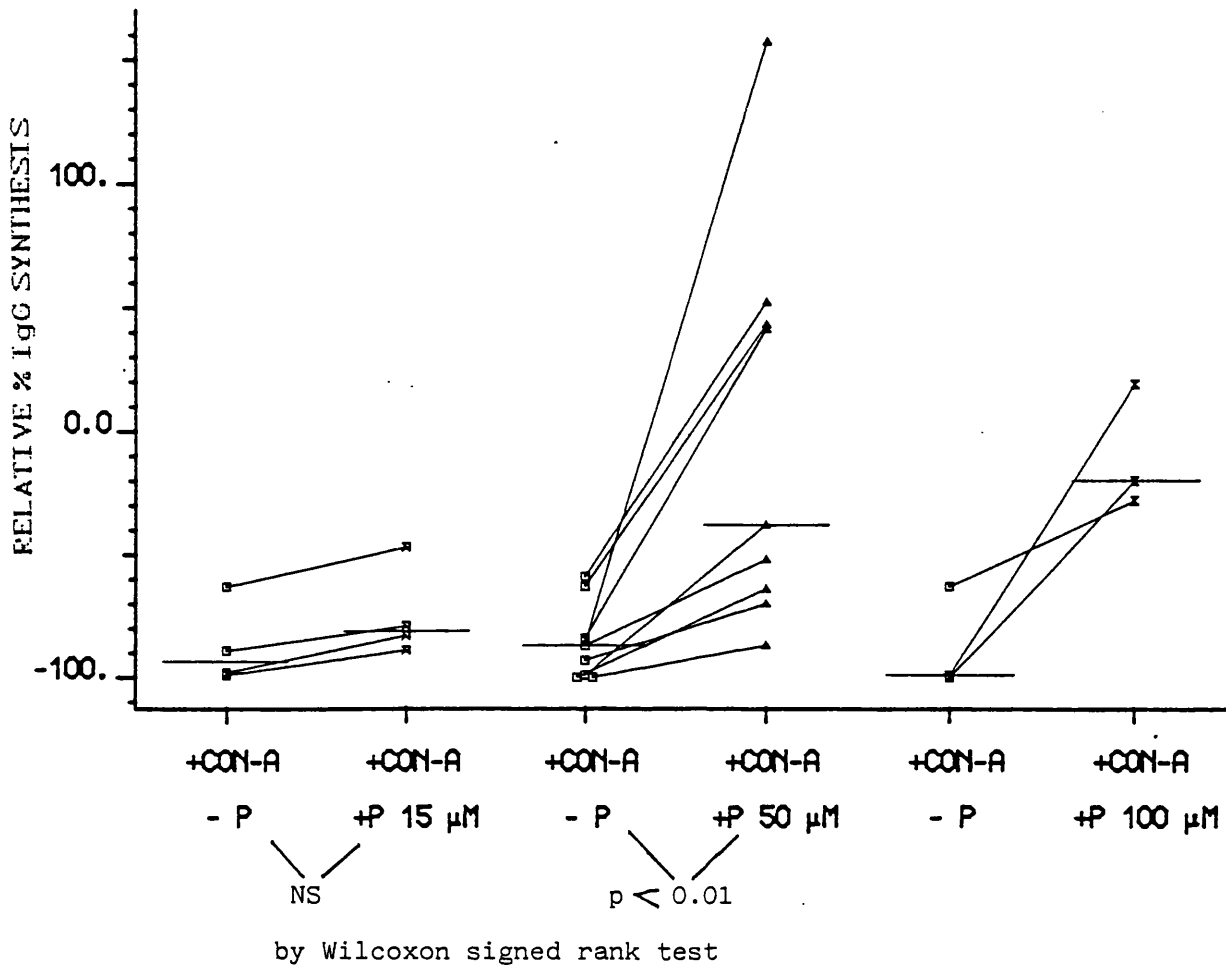
FIG. 4.46 EFFECT OF DILUTION OF EFFECTOR CELLS +/- PHMPA PRETREATMENT, ADDED TO A CONSTANT DENSITY OF RESPONDER CELLS ON RELATIVE % IgG SYNTHESIS



Points represent mean for n = 8 individuals; SEM were omitted for clarity.

FIG. 4.47

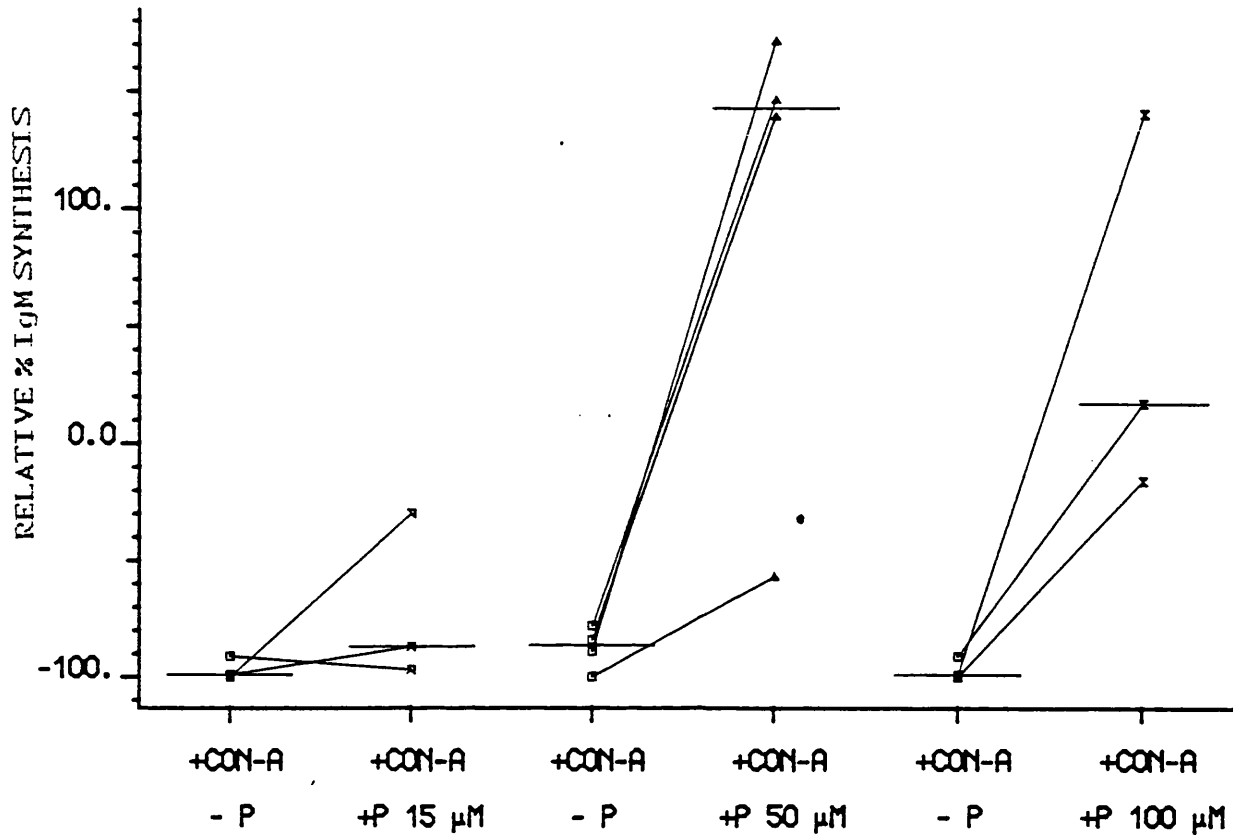
EFFECT OF POST-TREATMENT WITH PHMPA ON "ACTIVITY"
OF CON-A INDUCED SUPPRESSOR CELLS



Horizontal bars represent median relative % IgG synthesis.

FIG. 4.48

EFFECT OF POST-TREATMENT WITH PHMPA ON "ACTIVITY" OF
CON-A INDUCED SUPPRESSOR ACTIVITY ON IgM SYNTHESIS



Horizontal bars represent median relative % IgM synthesis.

suppressor activity to the point of actually stimulating IgG synthesis. Similarly, in the IgM assay, 1 out of 4 subjects showed a small effect following 50 μ M PHMPSA treatment, but 3 out of 4 showed a dramatic inhibition of suppressor activity to the point of actually stimulating IgM synthesis. In both activity and induction experiments appropriate controls consisted of untreated cells, pre- or post-treated with PHMPSA in a similar manner to the CON-A treated cells.

It was wondered whether lack of sensitivity to thiol blockade in the CON-A induced suppressor activity for IgG synthesis correlated with lack of thiol sensitivity of CON-A induced suppressor activity in the IgM assay; however, unfortunately none of the 3 subjects showing most lack of sensitivity to thiol blockade were investigated in the IgM assay, however the two subjects showing relatively little sensitivity to thiol blockade in the IgG assay were also investigated in the IgM assay, as shown in table 4.25, which illustrates that a lack of thiol sensitivity in one assay does not necessary correlate with a lack of sensitivity in the other.

In all these experiments a 1:200 dilution of PWM was found to again be an optimal dilution of PWM to use both to see CON-A induced suppression of Ig synthesis and to see maximal differences after PHMPSA treatment (data not shown). However, the same message was obtained using suboptimal and superoptimal PWM dilutions, i.e. activity but not induction of suppressor cells is inhibited by PHMPSA treatment.

TABLE 4.25

COMPARISON OF EFFECT OF PHMPA POST-TREATMENT ON CON-A INDUCED SUPPRESSOR
ACTIVITY IN IgG AND IgM ASSAYS IN 2 SUBJECTS.

	+CON-A NO PHMPA	+CON-A +50 μ M PHMPA
RELATIVE % IgG SYNTHESIS		
SUBJECT A	- 99%	- 38%
SUBJECT B	- 87%	- 52%
RELATIVE % IgM SYNTHESIS		
SUBJECT A	- 100%	- 57%
SUBJECT B	- 89%	171%

Visually it was observed in normals that UNT precultures contained well separated, evenly spaced cells, very few of which "clustered" together (Fig. 4.49). However, in the CON-A treated precultures numerous clusters were visible (Figs. 4.50-4.51) and cluster size depended on the dose of CON-A added, with larger clusters forming as a result of larger CON-A doses. PHMPSA pretreatment usually did not inhibit the tendency to cluster together; in fact in some wells the clusters were larger, but in some smaller. Likewise, a previous worker in our laboratory found PHMPSA treatment did not inhibit cluster formation [12]. PHMPSA pretreatment had visually no effect on the UNT preculture.

However, in the CON-A treated cocultures that had been post-treated with PHMPSA, fewer clusters were visible, and the cell population looked very similar to the UNT cocultures. When not-PHMPSA treated, CON-A cocultures contained numerous large cell clusters and the UNT coculture contained fewer and smaller clusters of cells.

In the RA NSAID cultures, clusters were seen in the 48 hour CON-A precultures, but there was improved clustering in the cocultures following 2-ME treatment.

In all cases the CON-A cocultures were more orange/yellow than the UNT cocultures at the end of a week's cultures, and this parallels the greater loss of viability of cells in these cultures than in the UNT cocultures (table 4.13), indicating a greater metabolic activity in the CON-A cocultures.

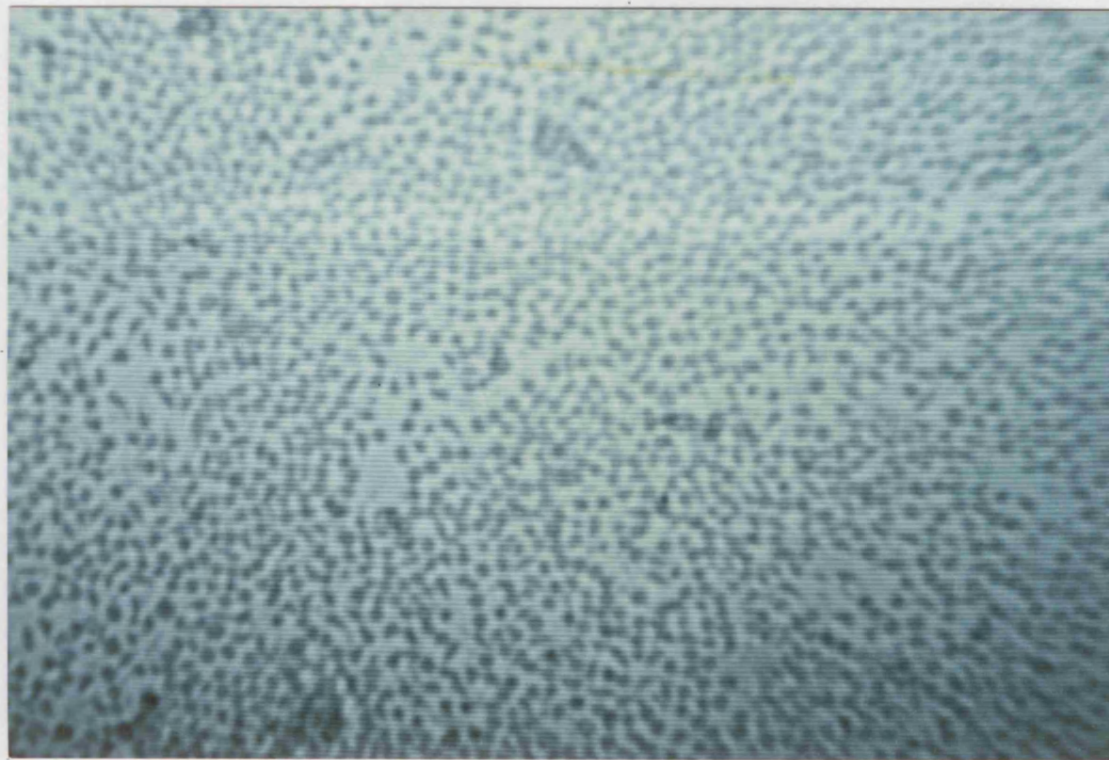


Fig. 4.49 : UNTREATED CELL POPULATION AFTER 48 HOUR PRECULTURE IN THE CON-A SUPPRESSOR CELL ASSAY. MAGNIFICATION X 100. PHOTO FROM VIDEO SCREEN.

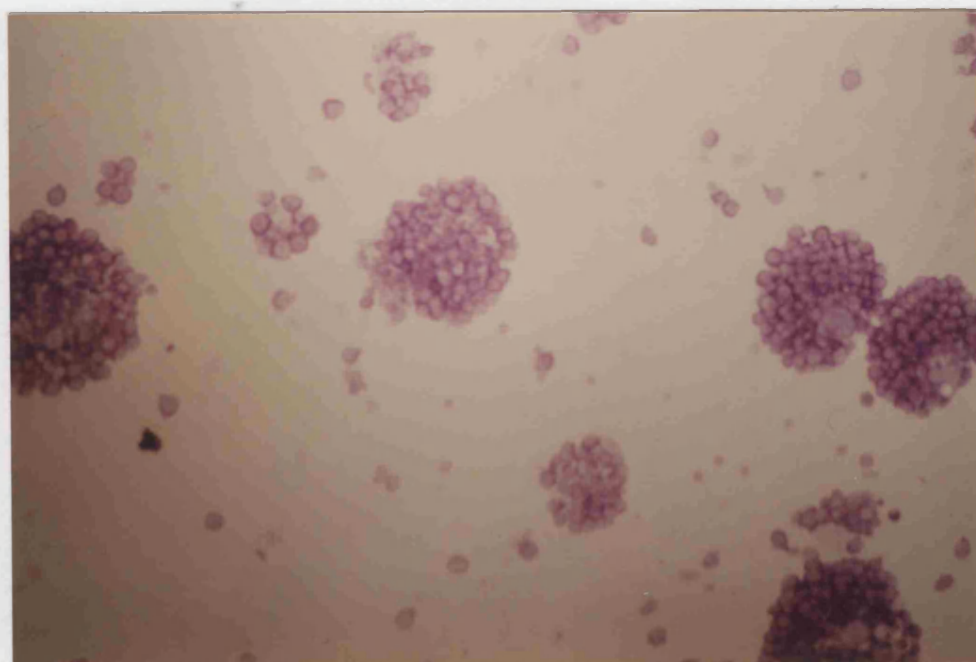


Fig. 4.50 : CON-A Rx CELL POPULATION AFTER 48 HOUR PRECULTURE IN THE CON-A INDUCED SUPPRESSOR CELL ASSAY. MAGNIFICATION X 100.

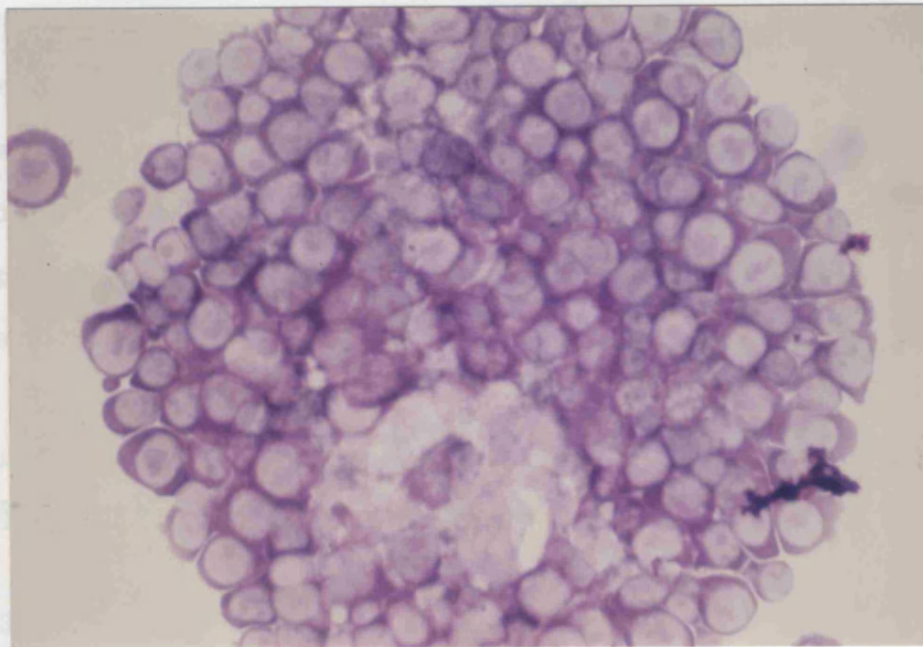


Fig.4.51 : CON-A TREATED CELLS AFTER 48 HOUR PRECULTURE IN THE CON-A INDUCED SUPPRESSOR ASSAY VIEWED UNDER HIGH POWER + OIL. MAGN. X 1250.

Having shown that CON-A induced suppressor activity is more sensitive to thiol blockade than is the induction of CON-A induced suppressor cells, we sought to see whether the defective suppressor activity seen in RA NSAID, which may be due to thiol oxidation, could be improved by 2-ME to reconvert disulphide bonds to their native free SH state.

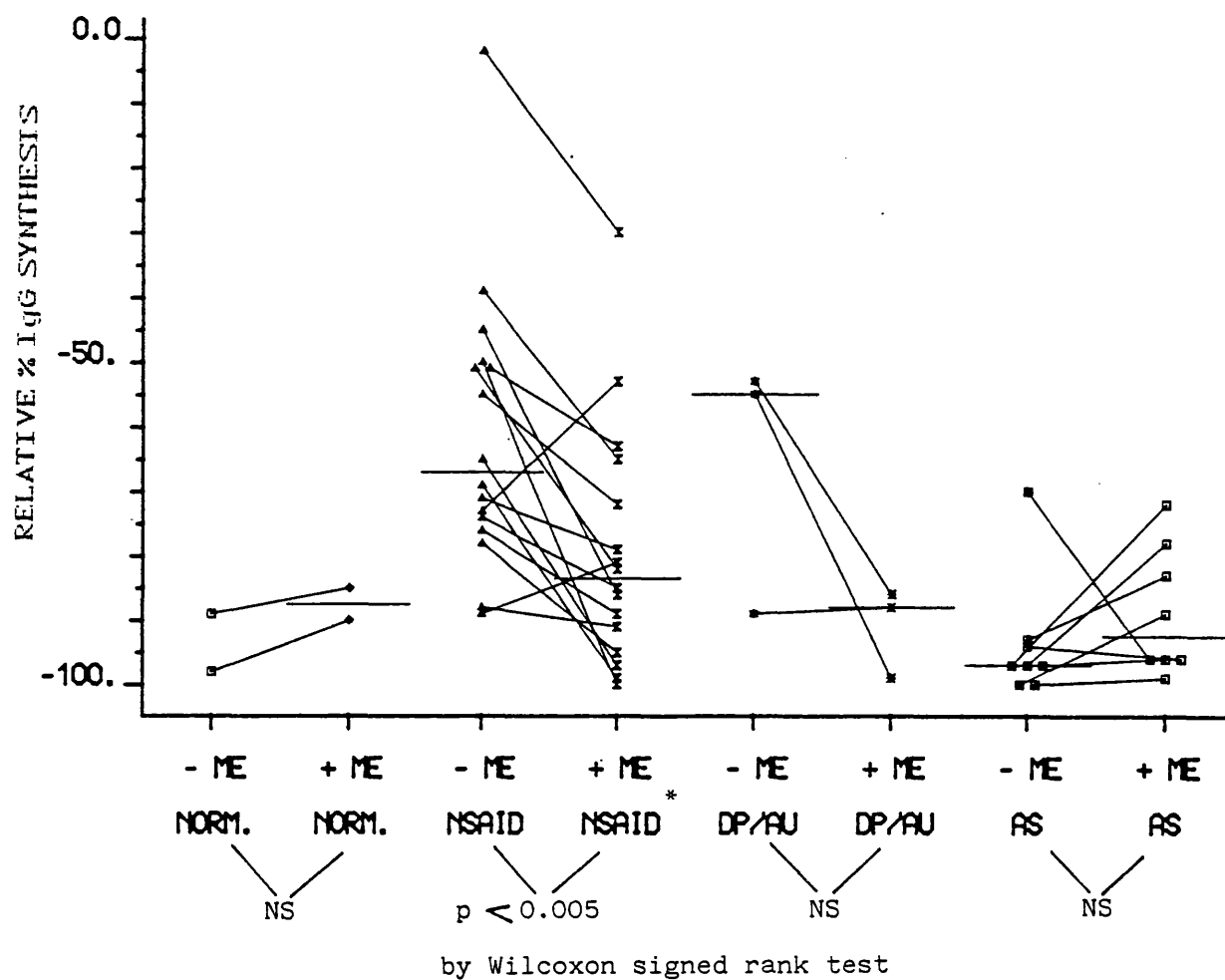
4.14. EFFECT OF 2-ME TREATMENT ON CON-A INDUCED SUPPRESSION IN RA NSAID PATIENTS.

Since the activity rather than induction of suppressor cells seems to be more thiol sensitive, it was decided more logical to assume that in RA NSAID patients there is no defect in induction of suppressor cells but that the activity is subsequently inhibited by cell surface -SH oxidation and as such it seemed more logical to treat the putative suppressor cells with 50 μ M 2-ME for 1 hour at 37 °C, prior to coculturing them with responder cells. To check that any effect observed was specific to RA NSAID and not a general phenomenon, cells were treated in a similar way in other patient groups.

Fig. 4.52 shows that there was a significant improvement ($p < 0.005$) in CON-A induced suppressor activity for IgG synthesis in RA NSAID after 2-ME treatment, but no effect in normals and AS patients. The improvement in RA NSAID was sufficient to normalise suppressor activity and this was also true for 2 out of 3 patients on DPEN/AU who originally showed defective suppressor activity. The third patient on DPEN showing normal suppression activity originally was not affected

FIG. 4.52

EFFECT OF POST-TREATMENT WITH 2-ME ON CON-A INDUCED SUPPRESSOR ACTIVITY ON IgG SYNTHESIS



* = NS vs. relative % IgG synthesis in $n = 11$ normals (median = -89%)

whose cells were incubated for 1 hour and washed after suppressor-cell generation.

Horizontal bars represent median relative % IgG synthesis.

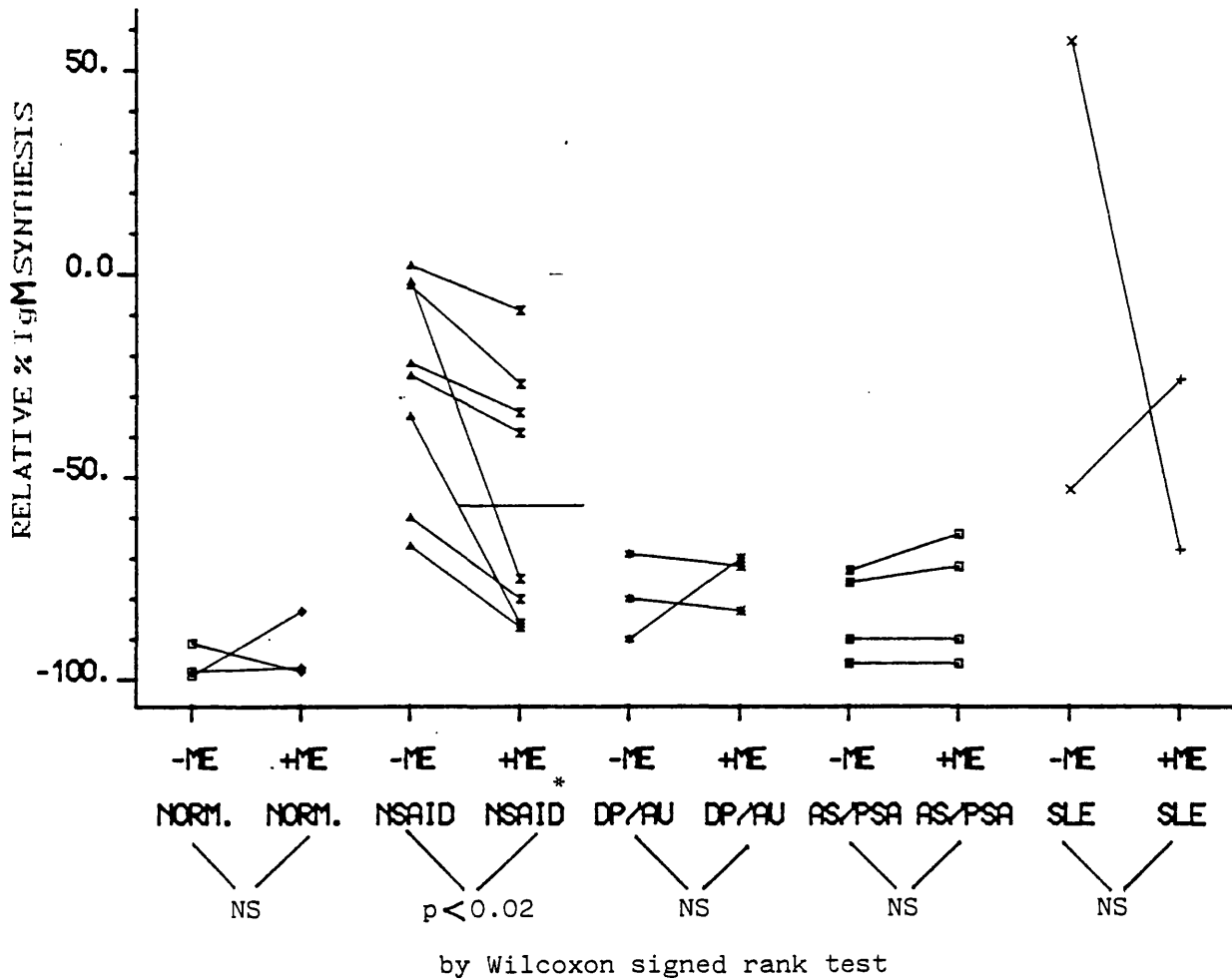
by this treatment. One of the 2 RA-DPEN/AU patients who were originally defective in suppressor activity had been on DPEN less than 6 months; in fact only for 4 months, but the other patient had been on intramuscular injections of aurothiomalate for 3 years. One must guess that the first subject taking DPEN had not been on treatment long enough for any beneficial effect to be seen and the person taking gold, who was said to be benefitting from it, should be changed to a different treatment or an increased dose of aurothiomalate.

Fig. 4.53 similarly shows that post-treatment with 2-ME significantly improved CON-A induced suppressor activity for IgM synthesis in RA NSAID compared to without 2-ME treatment ($p < 0.02$) but did not normalise it and there was no significant effect on AS/PSA or normal subjects. Not surprisingly there was no significant difference in CON-A induced suppressor activity after 2-ME treatment in RA-DPEN/AU subjects, since they were already showing normal suppressor activity. There was a suggestion of an improvement in suppressor activity in the SLE patients shown, but there was too little data to comment on.

Since it is not correct to assume that effects observed in normal subjects apply directly to the situation in RA NSAID, it was thought wise to analyse the effect of leaving 2-ME in the CON-A suppressor cell generating system in RA NSAID, to see if addition here too improved suppressor activity. Any ameliorating effect, if it occurs, could conceivably occur within the last hour of the 48 hour culture and would than be comparable to data in figs. 4.47 and 4.48. Again, fig. 4.54 shows 2-ME did significantly improve CON-A induced

FIG. 4.53

EFFECT OF POST-TREATMENT WITH 2-MERCAPTOETHANOL ON CON-A
INDUCED SUPPRESSOR ACTIVITY ON IgM SYNTHESIS

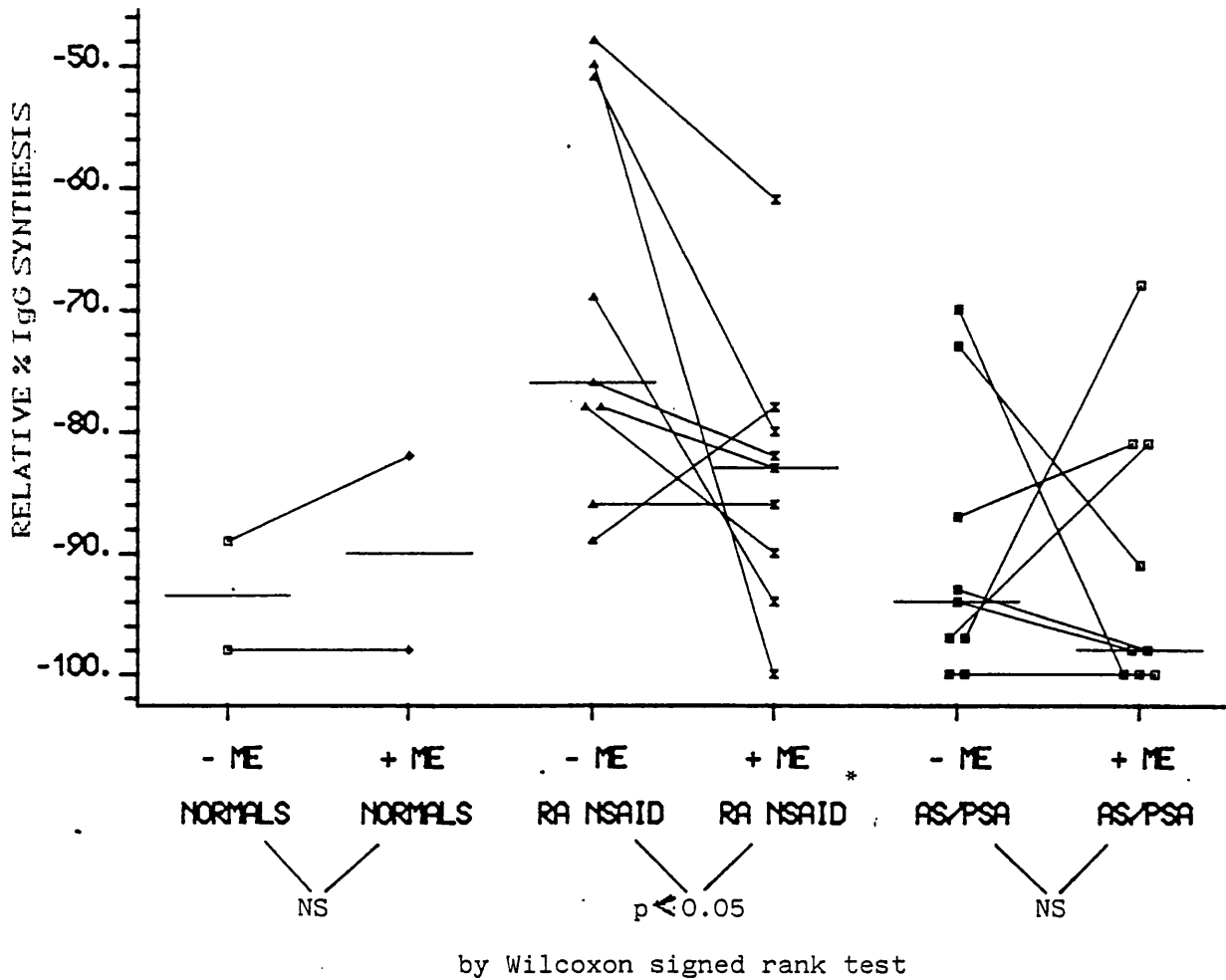


* = $p = 0.00001$ vs. relative % IgM synthesis in $n = 6$ normals (median = -90%) whose cells were incubated for 1 hour and washed after suppressor-cell generation.

Horizontal bars represent median relative % IgM synthesis.

FIG. 4.54

EFFECT OF LEAVING 2-ME IN CON-A SUPPRESSOR CELL GENERATING SYSTEM ON SUBSEQUENT SUPPRESSION OF IgG SYNTHESIS



* = NS vs. relative % IgG synthesis in $n = 19$ normals (median -90%)

whose cells were incubated for 1 hour and washed before suppressor-cell generation.

Horizontal bars represent median relative % IgG synthesis.

suppressor activity for IgG synthesis in RA NSAID subjects

($p < 0.05$), and did normalise it, but had no effect in normals and AS/PSA patients.

Similarly, 2-ME significantly improved CON-A induced suppressor activity for IgM synthesis in RA NSAID subjects compared to normals ($p < 0.05$, fig. 4.55), but did not normalise it and had no effect in RA-DPEN/AU, AS/PSA and normal subjects, who already showed normal suppressor activity.

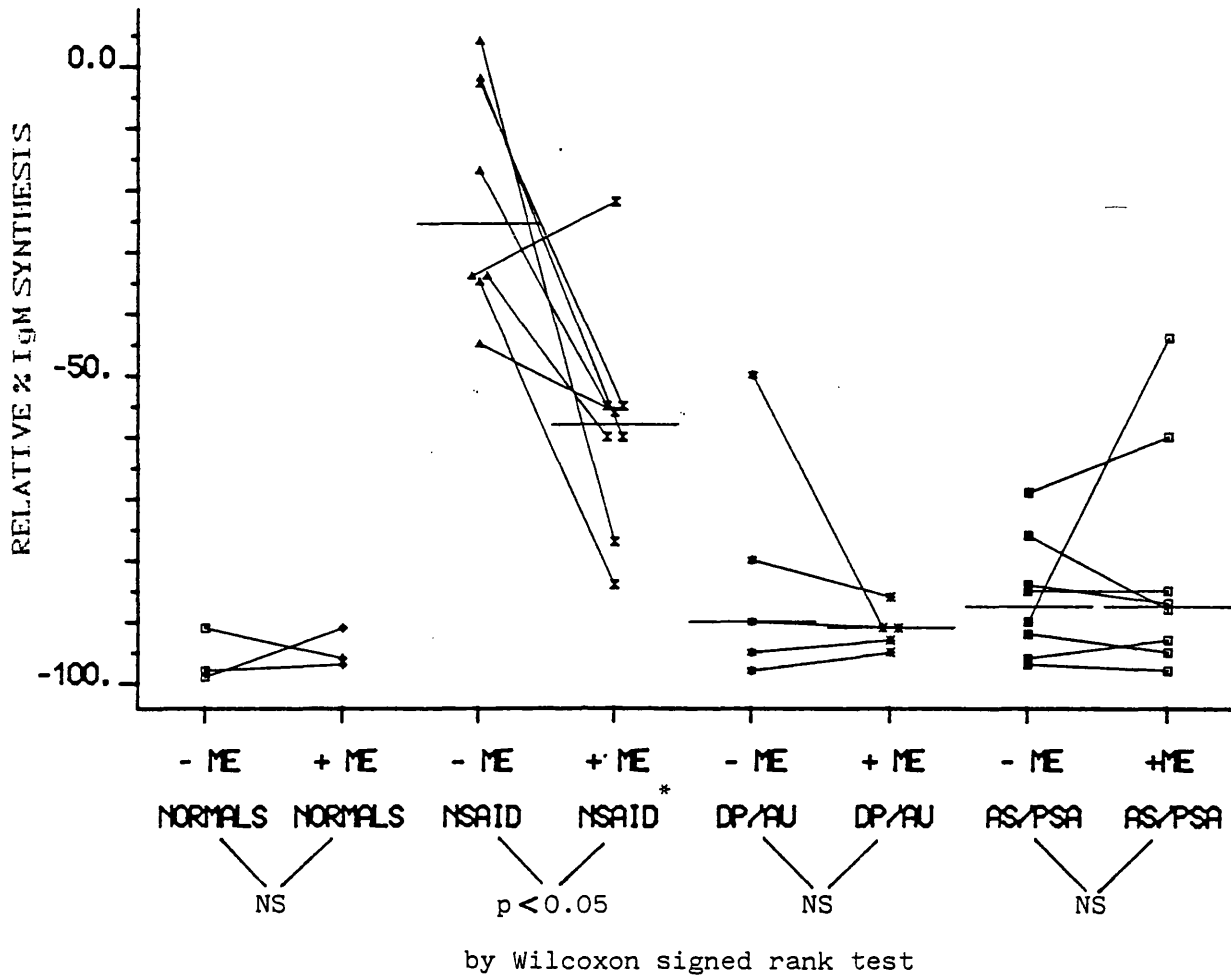
Pretreatment of PBMNC for 1 hour at 37 °C with 2-ME before CON-A stimulated generation of suppressor cells did not significantly improve suppressor cell activity in RA NSAID patients and had no effect in normal, RA-DPEN and AS patients, whether in the IgG or IgM assay (data not shown).

Table 4.26 shows one RA NSAID patient in whom 3 suppressor assays were utilized, and the effects of 2-ME treatment on suppressor activity in the CON-A induced suppressor systems were analysed. The 3 suppressor assays consisted of the CON-A induced suppressor assay using suppression of IgG or IgM synthesis and suppression of proliferation as a measure of successful suppressor cell generation, and the short lived suppressor assay where a high S.I. is indicative of the loss of spontaneous suppressor cells.

In this particular patient suppression of IgG synthesis was fairly good, but she showed defective suppression of IgM synthesis, and the S.I. was low, indicating defective short lived suppressor cells. 2-ME treatment had a more profound effect on improving the

FIG. 4.55

EFFECT OF LEAVING 2-ME IN CON-A SUPPRESSOR CELL GENERATING SYSTEM ON SUBSEQUENT SUPPRESSION OF IgM SYNTHESIS



* = $p < 0.001$ vs. relative % IgM synthesis in $n = 9$ normals (median = -95%) whose cells were incubated for 1 hour and washed before suppressor-cell generation.

Horizontal bars represent median relative % IgM synthesis.

TABLE 4.26

ONE 82 YEAR OLD FEMALE PATIENT OF DISEASE DURATION = 22 YEARS ON NSAID THERAPY
WITH ACTIVE SYNOVITIS AND EXTRAARTICULAR DISEASE (SJOEGREN'S SYNDROME) WAS
STUDIED IN DETAIL USING 3 DIFFERENT SUPPRESSOR CELL ASSAYS.

SERUM - SH	CON-A INDUCED SUPPRESSOR ACTIVITY ASSESSED AS RELATIVE % IgG SYNTHESIS (10 µg/ml CON-A)	CON-A INDUCED SUPP. ACTIVITY ASSESSED AS RELATIVE % IgM SYNTHESIS (10 µg/ml CON-A)	CON-A INDUCED SUPP. ACTIVITY ASSESSED AS RELATIVE % PROLIFRATION IN RESPONSE TO PHA 0.1% V:V	SHORT-LIVED SUPPRESSOR CELL ASSAY STIMULATED WITH 5 µg/ml CON-A AS ASSESSED BY SI
312	- 78 %	- 45 %	- 36 %	1.03
<p>ADDITION OF 2ME FOR 48 h WITH CON-A SUPPRESSOR CELL GENERATING SYSTEM</p> <p>- 83 %</p>			<p>EFFECT OF 2ME TREATMENT OF CELLS ON SUBSEQUENT SUPPRESSION</p> <p>- 80%</p>	
<p>IN ALL CASES ONLY RESPONDER CELLS MADE Ig's AND UNTREATED AND CON-A TREATED CELLS ALONE PRODUCED INSIGNIFICANT Ig's LEVELS.</p>			<p>MYTOMICIN-C TREATMENT OF UNTREATED CELLS AND CON-A TREATED CELLS INHIBITED THEIR SUBSEQUENT PROLIFERATION IN RESPONSE TO PHA, SO ONLY RESPONDER CELLS PROLIFERATE</p>	
<p>ONLY ADDITION OF CON-A TREATED CELLS TO RESPONDER CELLS RESULTED IN REDUCED Ig's SYNTHESIS FROM THEM.</p>			<p>ONLY ADDITION OF CON-A TREATED CELLS, NOT UNTREATED CELLS, TO RESPONDER CELLS INHIBITED PROLIFERATION OF RESPONDER CELLS</p>	
COMPARED TO DATA IN NORMALS :				
n = 42		n = 20		n = 6
MEAN = - 86 %		MEAN = - 93 %		MEAN = 2.5

significantly defective suppression of proliferation compared to the improvement in suppression of Ig's synthesis; not surprisingly 2-ME caused greater improvement in suppression of IgM synthesis than IgG, the former being more defective in the first place. Thus, this patient showed simultaneous dysfunction in several suppressor cell circuits.

In three other RA NSAID patients with active synovitis in whom CON-A induced suppression of proliferation only was investigated, a median percent proliferation of -38% was observed and, similarly to the above patients, 2-ME treatment normalised the suppression. In four normals in whom CON-A induced suppression of proliferation was investigated, a median suppression of 81% was observed, with a range 60-99%. The degree of suppression seemed more variable using this assay, which was another reason for preferring to look at suppression of PWM-stimulated immunoglobulin synthesis.

4.15. EFFECT OF H_2O_2 ON CON-A INDUCED SUPPRESSION.

Since PHMPA is not a physiological thiol blocking agent and was used simply because it is non reversible, thus it aids interpretation of data which might be missed if an agent were used that was reversible, it was wondered whether similar results might be obtained using the physiological oxidizing agent H_2O_2 . It was known that the interpretation of such data would need to be given with caution because H_2O_2 is readily reduced in the air and by the enzymes both intra and extracellularly, e.g. catalase and the glutathione peroxidase enzymes; thus a lack of effect using it may not mean that there had been no effect. Also it must be remembered that cells have

the capacity to reconvert disulphide bonds to free SH groups via the glutathione redox cycle, involving the enzymes glutathione peroxidase and its essential co-substrate glutathione and glutathione reductase. However, cells do not have the capacity to reverse the effect of PHMPSA, since it does not cause disulphide linkages but actually blocks SH groups by binding to them with its mercury moiety.

Since in vivo it can be envisaged that H_2O_2 would be produced constantly during an inflammatory active synovitis episode, it was thought more physiological to leave H_2O_2 both in the 48 hour CON-A suppressor cell generating system and the subsequent coculture.

Fig. 4.56 shows that, when added to cells from normal subjects, increasing doses of H_2O_2 caused an increased loss of suppressor activity with considerable loss of activity even at 10 nM H_2O_2 . Similar results were obtained using AS patients (data not shown). It must be recorded that low doses of H_2O_2 were found to be mitogenic for IgG synthesis as shown in fig. 4.19.

Fig. 4.57 shows that paired data at 10 nM and 10 μ M H_2O_2 in normals and AS subjects although not significantly different at either concentration do show a general trend of loss of suppressor activity following H_2O_2 treatment for the IgG assay. Fig. 4.58 suggests that RA - NSAID patients may be more sensitive to loss of suppressor activity by H_2O_2 than normals, since the activity falls off more rapidly. This may be because they already have oxidized thiol groups on some functionally important cell surface proteins, so a further exposure to an oxidizing agent could conceivably oxidize all remaining unoxidized

FIG.4.56 EFFECT OF H_2O_2 ON RELATIVE % IgG SYNTHESIS IN NORMAL SUBJECTS

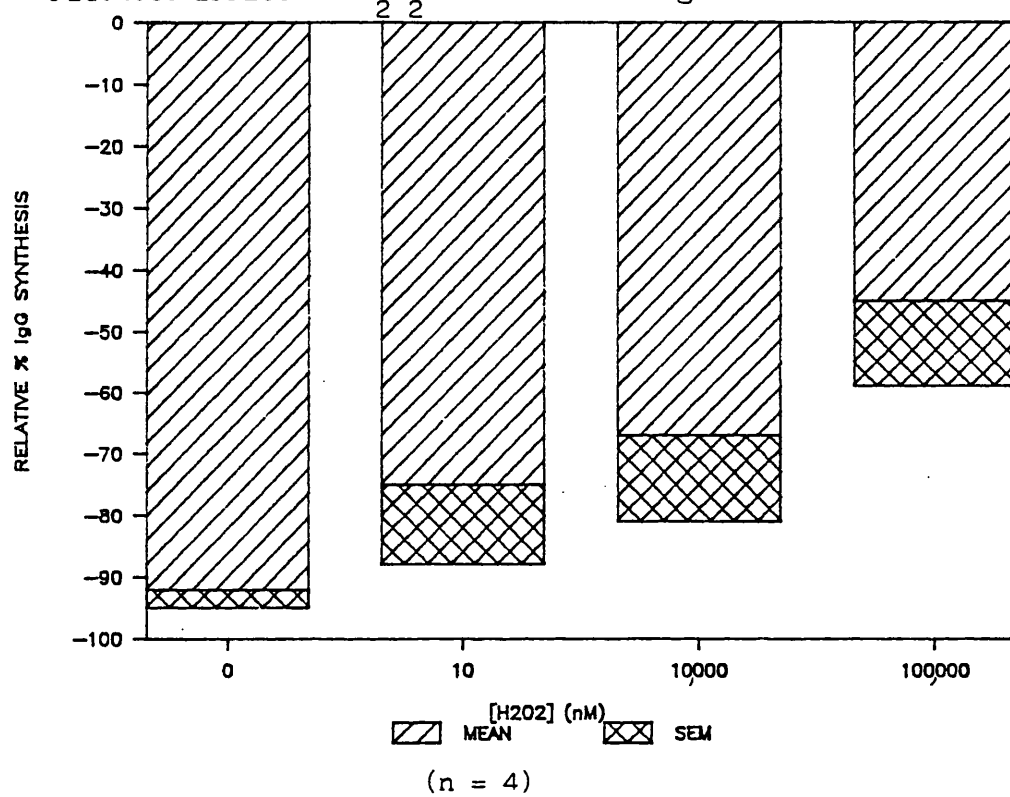
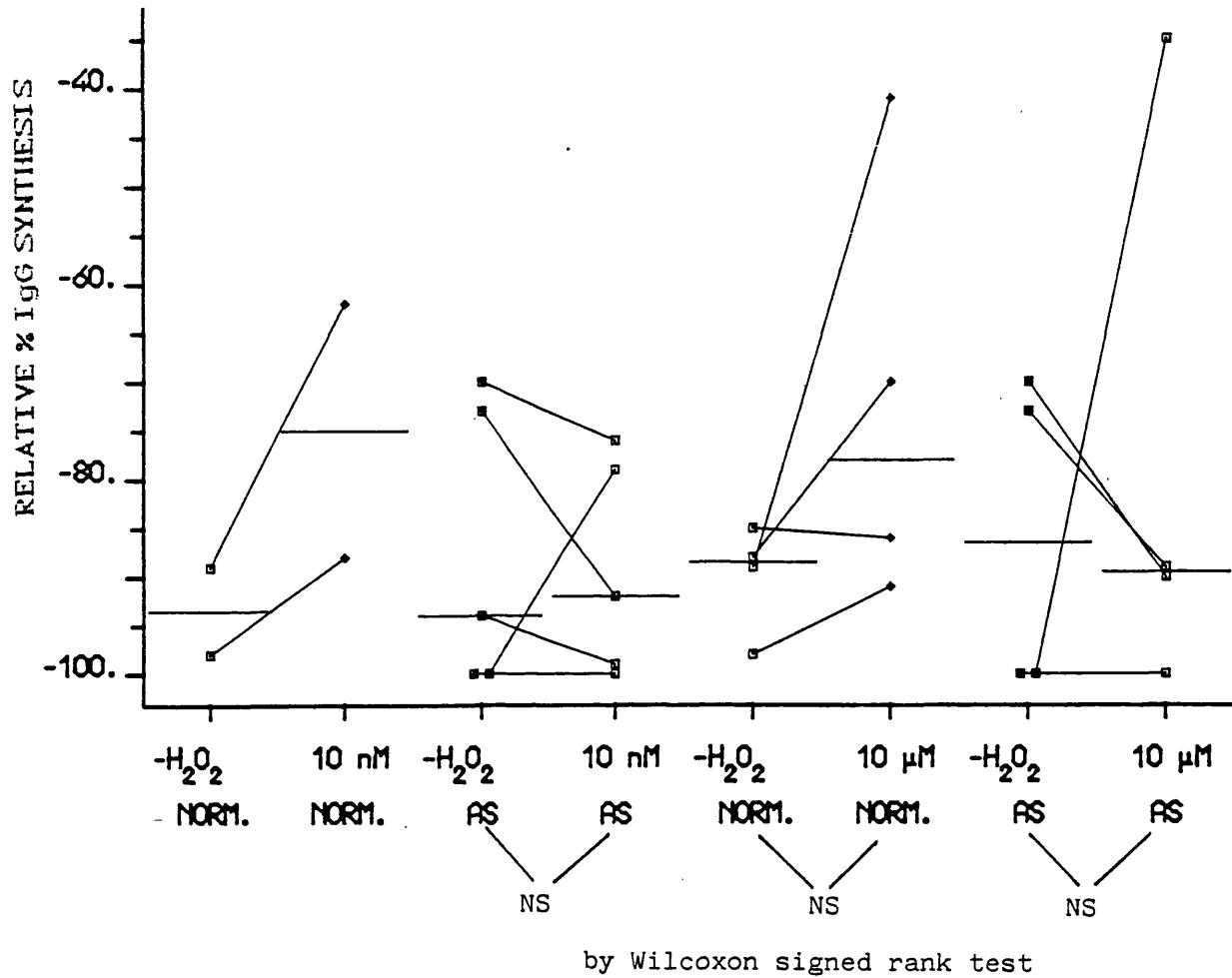


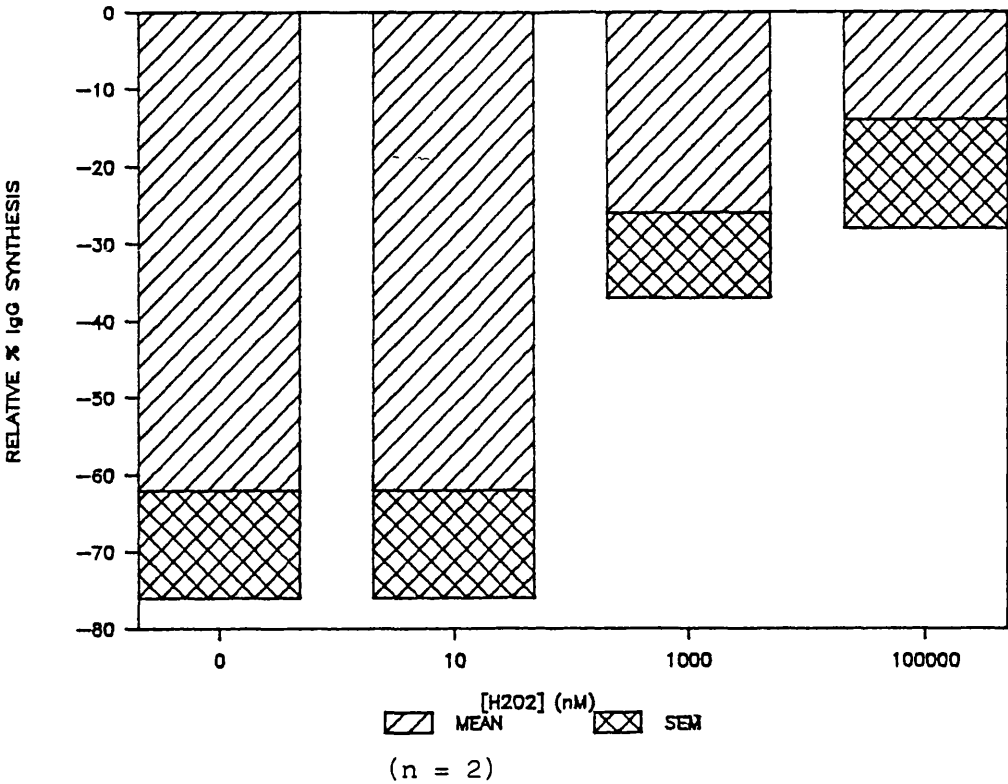
FIG. 4.57

EFFECT OF LEAVING H_2O_2 IN CULTURE ON CON-A INDUCED
SUPPRESSOR ACTIVITY ON IgG SYNTHESIS



Horizontal bars represent median relative % IgG synthesis.

FIG.4.58 EFFECT OF H₂O₂ ON RELATIVE % IgG SYNTHESIS IN RA NSAID PATIENTS



thiol groups.

Fig. 4.59 shows similar results in the IgM assay at $1\ \mu\text{M}$ and $10\ \mu\text{M}$ H_2O_2 for RA NSAID, DPEN/AU, AS and normal subjects.

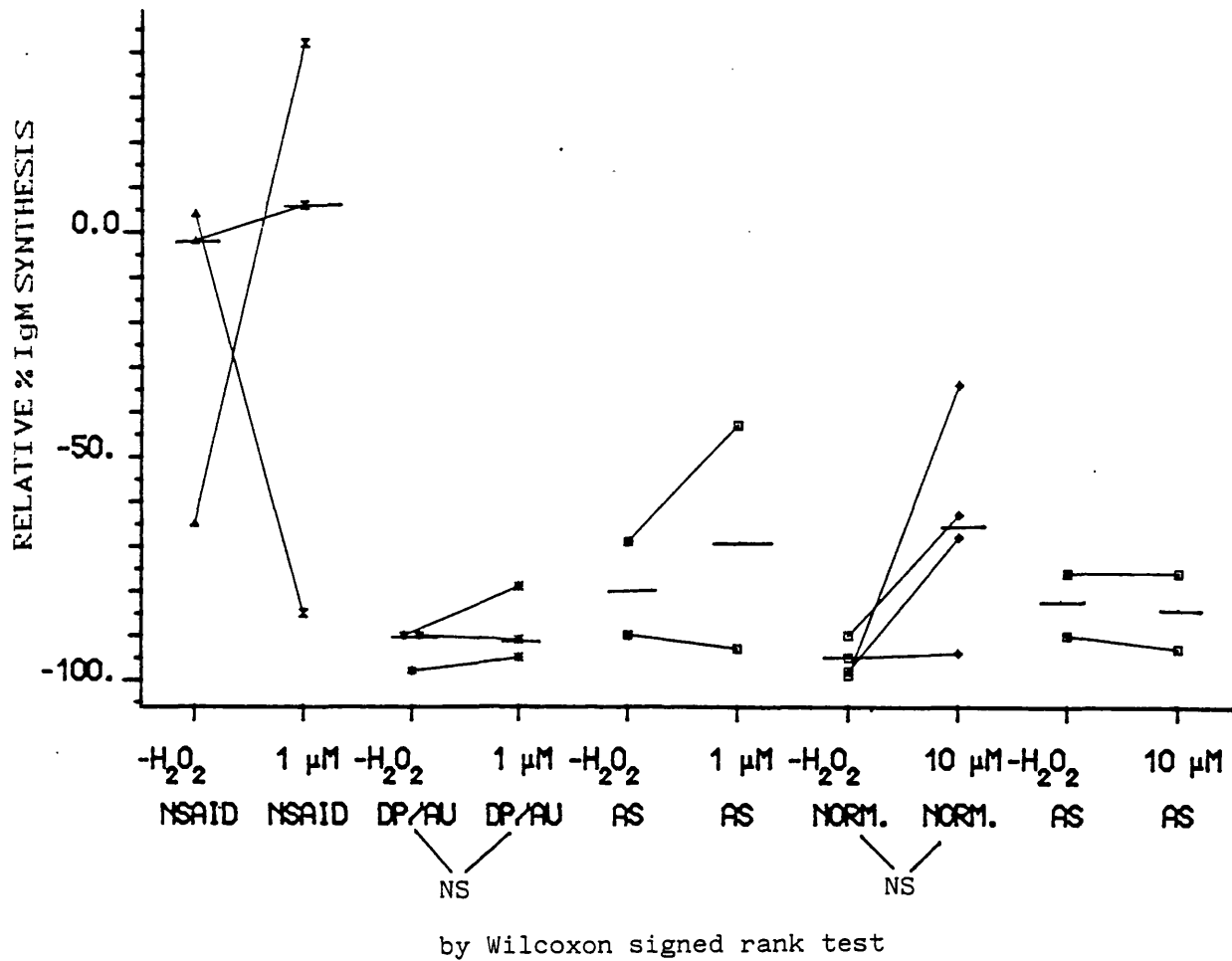
Thus the data obtained with H_2O_2 treatment, although less marked than with PHMPA, do show the same trend, that is CON-A induced suppressor activity is sensitive to thiol oxidation and thiol blockade and the two facts together would suggest that it is likely that the effect seen with both reagents parallel each other because it is the same functionally important cell surface molecules that are affected, namely thiol groups.

In view of this hypothesis it was thought appropriate to see if there was a correlation between CON-A induced suppressor activity for IgG and IgM synthesis and serum thiol groups, the latter being an indirect measurement of what might be happening to cell surface thiol groups. In order to obtain a range of thiol groups extending over a normal and an abnormal range, all subjects (i.e. normals, RA NSAID, RA-DPEN/AU and AS/PSA patients) were considered together on one graph.

Interestingly, there was a significant correlation ($r = 0.44$, $p < 0.01$) between relative percent of IgG synthesis and serum thiol levels (Fig. 4.60) and an even more significant correlation between relative percent of IgM synthesis and serum thiol levels (Fig. 4.61, $r = 0.64$, $p < 0.001$). There was an insufficient range of thiol levels within individual patient groups to do this correlation per patient group.

FIG. 4.59

EFFECT OF LEAVING H_2O_2 IN CULTURE ON CON-A INDUCED SUPPRESSOR ACTIVITY ON IgM SYNTHESIS



Horizontal bars represent median relative % IgM synthesis.

FIG. 4.60 CORRELATION OF RELATIVE % IgG SYNTHESIS VS. SERUM SH LEVELS
IN ALL SUBJECTS (NORMALS, AND ALL PATIENTS)

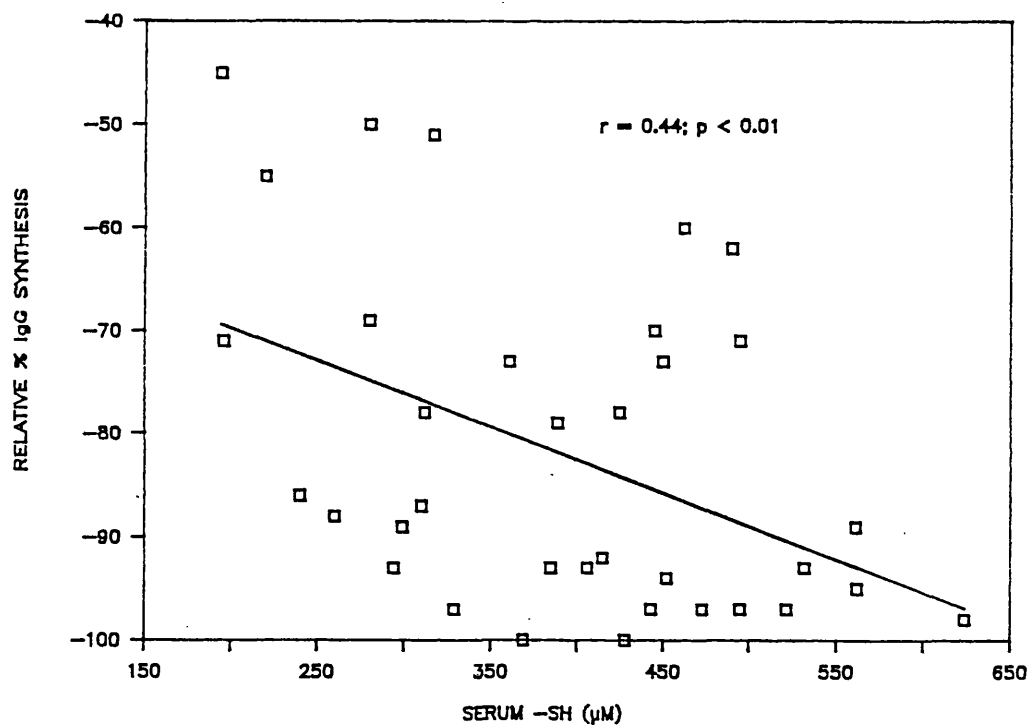
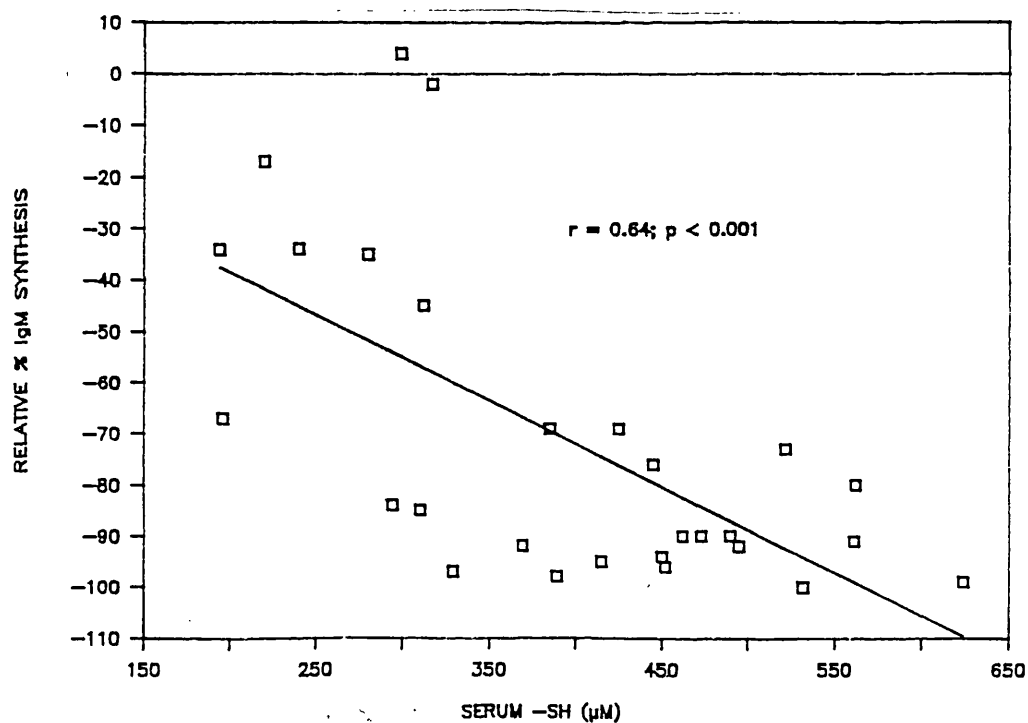


FIG. 4.61 CORRELATION OF RELATIVE % IgM SYNTHESIS VS. SERUM SH LEVELS
IN ALL SUBJECTS (NORMALS AND ALL PATIENTS)



4.16. EFFECT OF PHMPA ON MITOGEN STIMULATED PROLIFERATION.

The fact that CON-A induced suppressor activity was more sensitive to thiol blockade than induction was surprising in the light of experiments carried out on normal subjects showing proliferation in response to various mitogen, i.e. CON-A, PHA, anti CD3 Mab and PWM, was increasingly inhibited by increasing concentrations of PHMPA when the latter was left in the 72 hours culture (data not shown). However, such experiments could be criticised since it could be argued that the inhibitory effect was due to PHMPA interacting with and inhibiting important components in the medium, or even inhibiting the action of the mitogens themselves. This could be the interpretation of table 4.27, which illustrates viabilities of cells after 72 hours incubation +/- mitogen, +/- various concentrations of PHMPA, where it was found that viabilities fell to a greater extent in the presence of mitogens, than in their absence, and PHMPA further decreased viabilities in a dose dependent manner more significantly at 100 μ M PHMPA than at 50 μ M, but that even at 100 μ M viabilities were still acceptable.

To avoid this criticism, the experiments were carried out with pretreatment of PBMNC with 50 μ M PHMPA for 1 hour at 37 °C then washed off, followed by a 72 hours incubation with various mitogens. In this way viabilities were even less effected, even following 100 μ M PHMPA treatment, where viability was greater than 95%.

Initially experiments were set up to find the optimum dose of mitogen to use in these experiments. Fig. 4.62-4.65 show that

TABLE 4.27

CELL VIABILITY AFTER 72 HOUR INCUBATION WITH/WITHOUT MITOGEN,
WITH/WITHOUT VARIOUS PHMPA CONCENTRATION (RESULTS EXPRESSED AS %
VIABLE CELLS \pm SEM, P = PHMPA).

% VIABLE CELLS		% VIABLE CELLS		% VIABLE CELLS	
UNTREATED....	99 \pm 1	CON-A 10 μ g/ml....	82 \pm 3	PHA 0.1% V:V....	77 \pm 12
+ 5 μ M P.....	99 \pm 0.6	+ 5 μ M P.....	80	+ 5 μ M P.....	77 \pm 12
+ 10 μ M P....	99 \pm 0.5	+ 10 μ M P.....	81	+ 5 μ M P.....	73 \pm 8
+ 50 μ M P....	99 \pm 0.4	+ 50 μ M P.....	82	+ 50 μ M P.....	70 \pm 14
+ 100 μ M P...	99 \pm 1	+ 100 μ M P.....	79 \pm 3	+ 100 μ M P.....	61 \pm 20

TABLE 4.28

EXPERIMENT TO FIND THE OPTIMUM CONCENTRATION OF
2-MERCAPTOETHANOL (2-ME) TO MAXIMALLY IMPROVE PROLIFERATION
IN RA NSAID WITHOUT AFFECTING CELL VIABILITY ($n = 6$).

\pm 2-ME (μ M)	0	50	100	500
\bar{x} (CPM/ 10^6 -BKG)	268067	323756	241260	53613
\pm SEM	81092	91110	81000	1000
CELL VIABILITY	99%	99%	99%	30% (70% cell death)

FIG. 4.62 DOSE-RESPONSE CURVE FOR CON-A INDUCED PROLIFERATION
IN NORMAL SUBJECTS AFTER A 72 HOUR CULTURE PERIOD

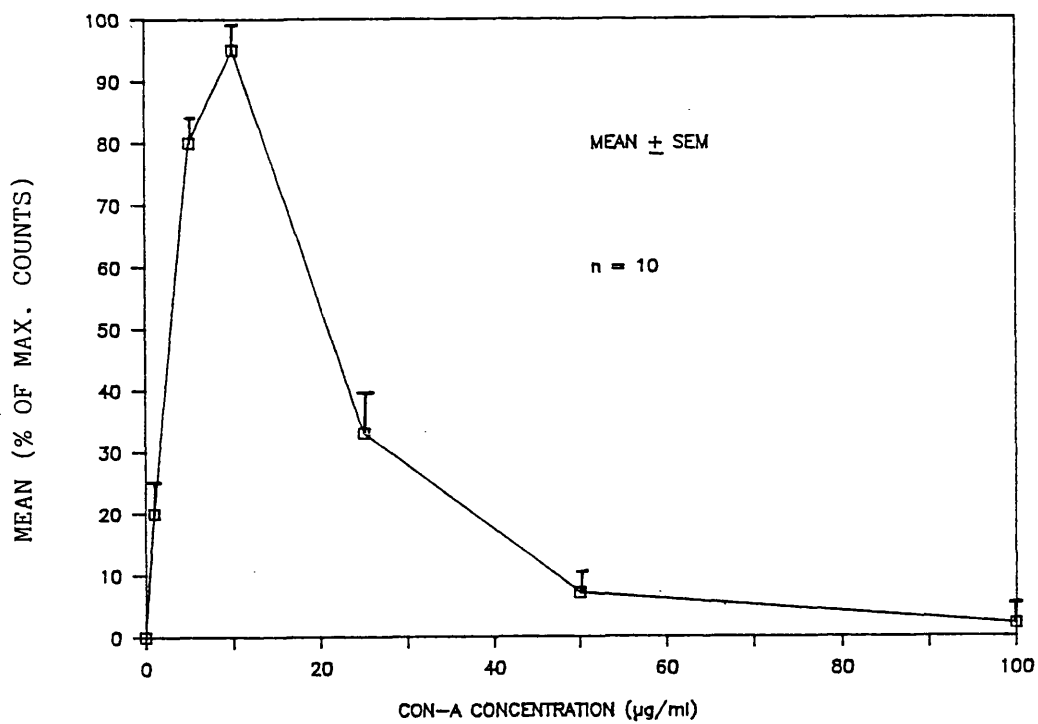


FIG. 4.63 DOSE-RESPONSE CURVE FOR PHA INDUCED PROLIFERATION
IN NORMAL SUBJECTS AFTER A 72 HOUR CULTURE PERIOD

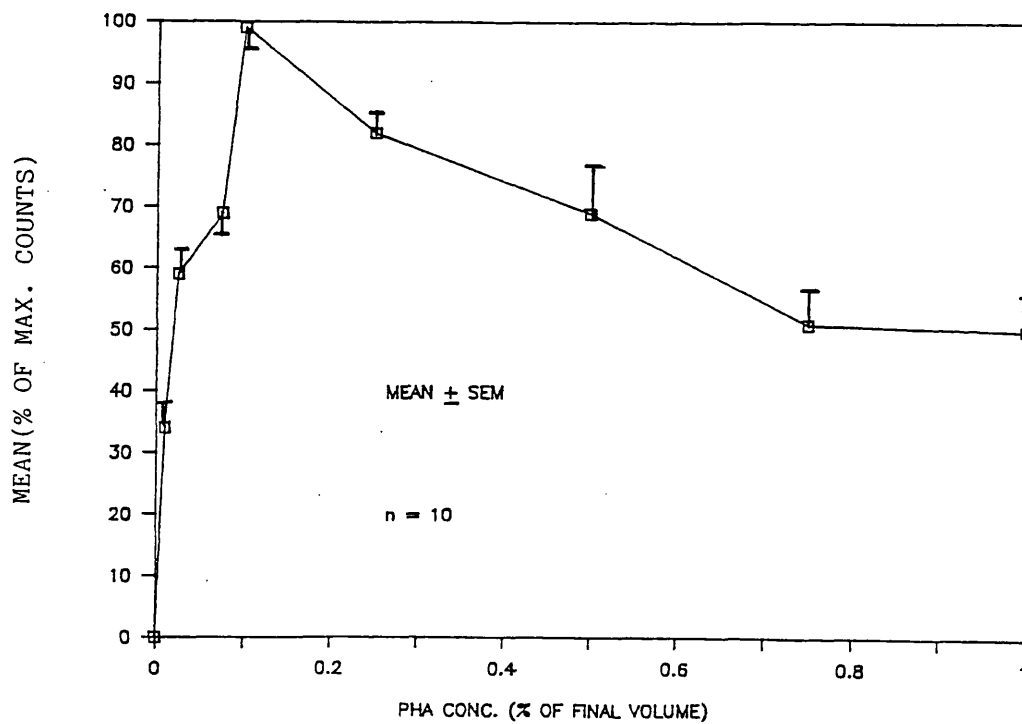


FIG. 4.64 DOSE-RESPONSE CURVE FOR Mab-T3 INDUCED PROLIFERATION

IN NORMAL SUBJECTS AFTER A 72 HOUR CULTURE PERIOD

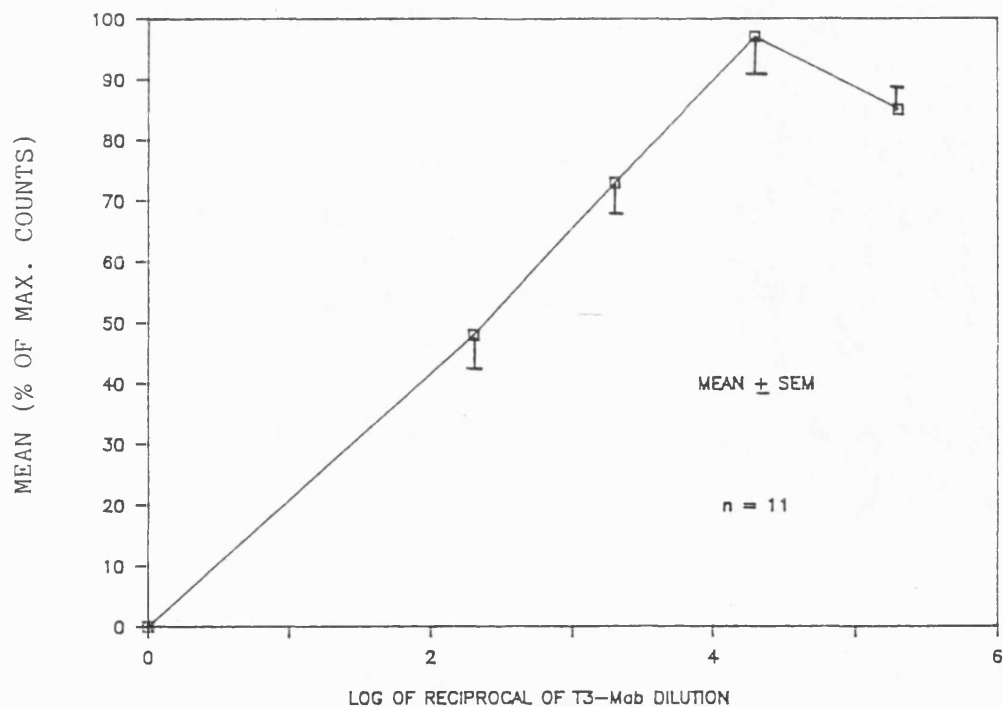
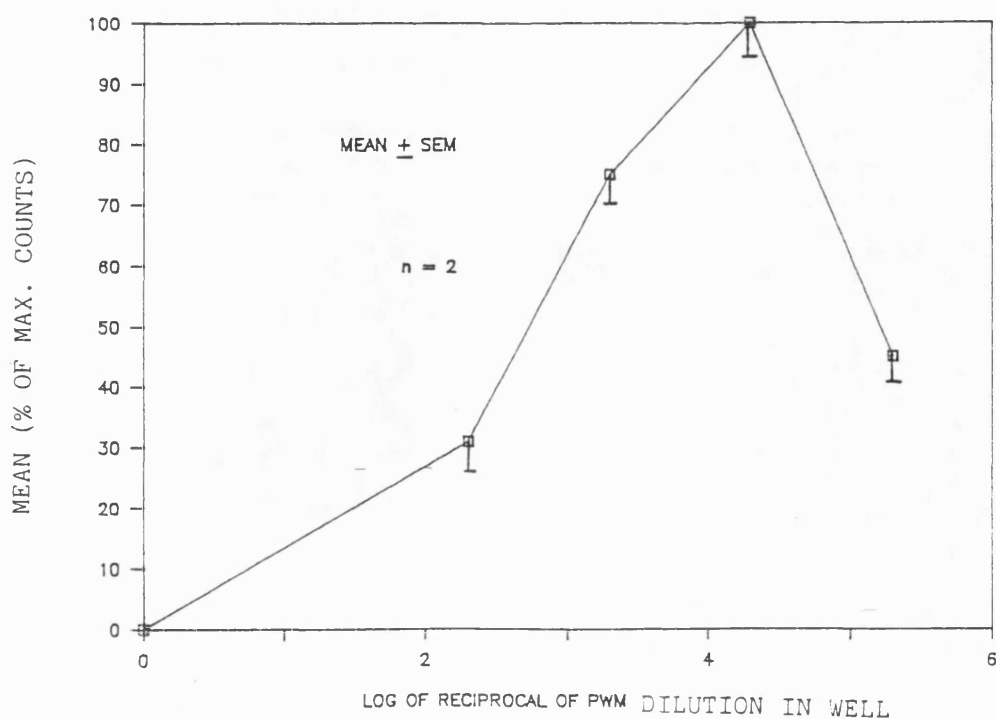


FIG. 4.65 DOSE-RESPONSE CURVE FOR PWM INDUCED PROLIFERATION

IN NORMAL SUBJECTS AFTER A 72 HOUR CULTURE PERIOD



respectively 10 $\mu\text{g/ml}$ CON-A, 0.1% v/v PHA (10 $\mu\text{g/ml}$), 1:20,000 dilution of anti CD3 Mab and 1:200 dilution of PWM were optimum doses per $1 \times 10^6/\text{ml}$ PBMNC in 200 μl culture. Results were expressed as percentage of maximum counts for each dose within an individual and then percentages for each dose averaged for all the normal individuals under investigation, because the actual CPM data varied considerably subject to subject, as seen in Figs. 4.70-4.72.

Figs. 4.66-4.69 show the effect of pretreatment with PHMPSA on subsequent proliferation; again because of the large individual variation in CPM it was thought wiser to normalise the data by expressing the results at each PHMPSA concentration as percentage of counts relative to those in a population of cells not treated with PHMPSA (per each individual), which were then averaged for all the normal subjects under investigation. Again, PHMPSA pretreatment was found to inhibit subsequent proliferation in response to the various stimuli.

4.17. EFFECT OF 2-ME TREATMENT ON PROLIFERATION OF RA NSAID CELLS.

The defective proliferation observed following mitogen treatment (CON-A, PHA and anti-CD3 Mab) in RA NSAID patients was found to be improved by a 1 hour preincubation with 50 μM 2-ME at 37 °C, found previously to be an optimum concentration for stimulation of proliferation without inhibiting cell viability (Table 4.28). Figs 4.70-4.72 show 2-ME significantly improved the proliferative response to CON-A ($p < 0.05$), to PHA ($p < 0.05$) and to anti-CD3 Mab ($p < 0.05$)

FIG. 4.66 EFFECT OF PRETREATMENT WITH PHMPA ON SUBSEQUENT PROLIFERATION
IN RESPONSE TO 10 $\mu\text{g/ml}$ CON-A IN NORMAL SUBJECTS

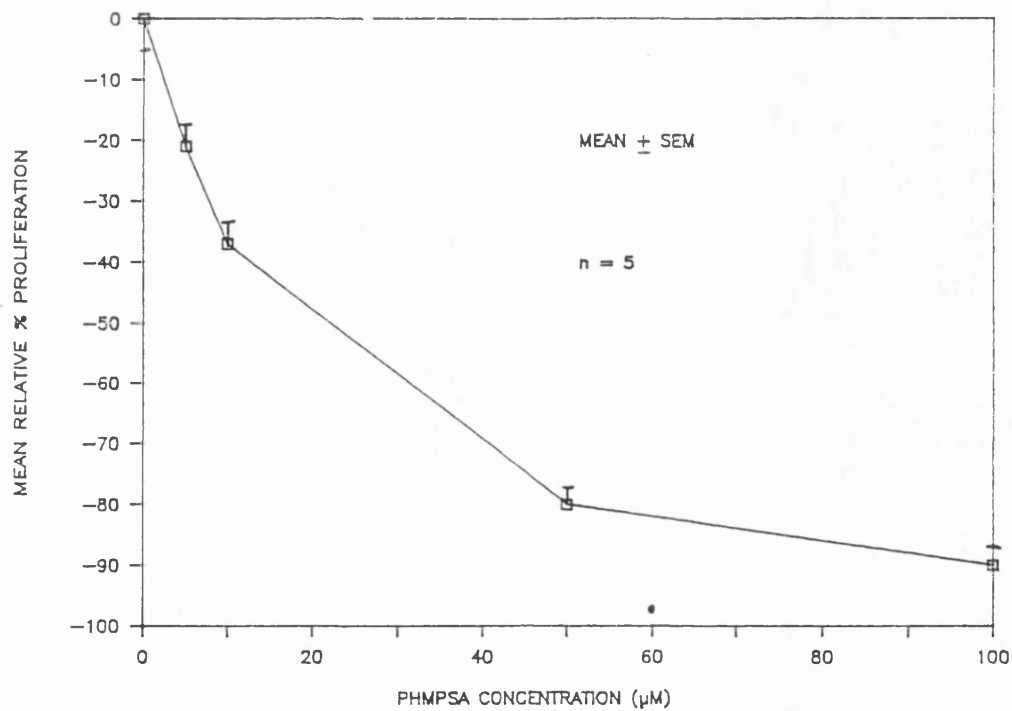


FIG. 4.67 EFFECT OF PRETREATMENT WITH PHMPA ON SUBSEQUENT PROLIFERATION
IN RESPONSE TO 0.1% V:V PHA IN NORMAL SUBJECTS

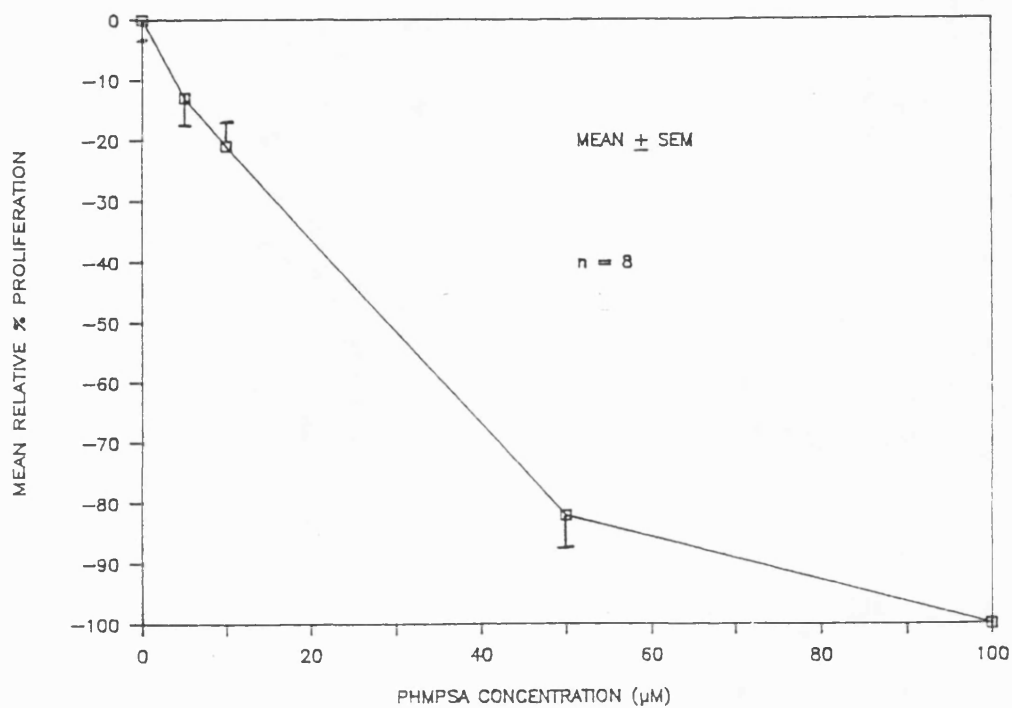


FIG. 4.68 EFFECT OF PRETREATMENT WITH PHMPA ON SUBSEQUENT PROLIFERATION
IN RESPONSE TO 1:20,000 Mab-T3 IN NORMAL SUBJECTS

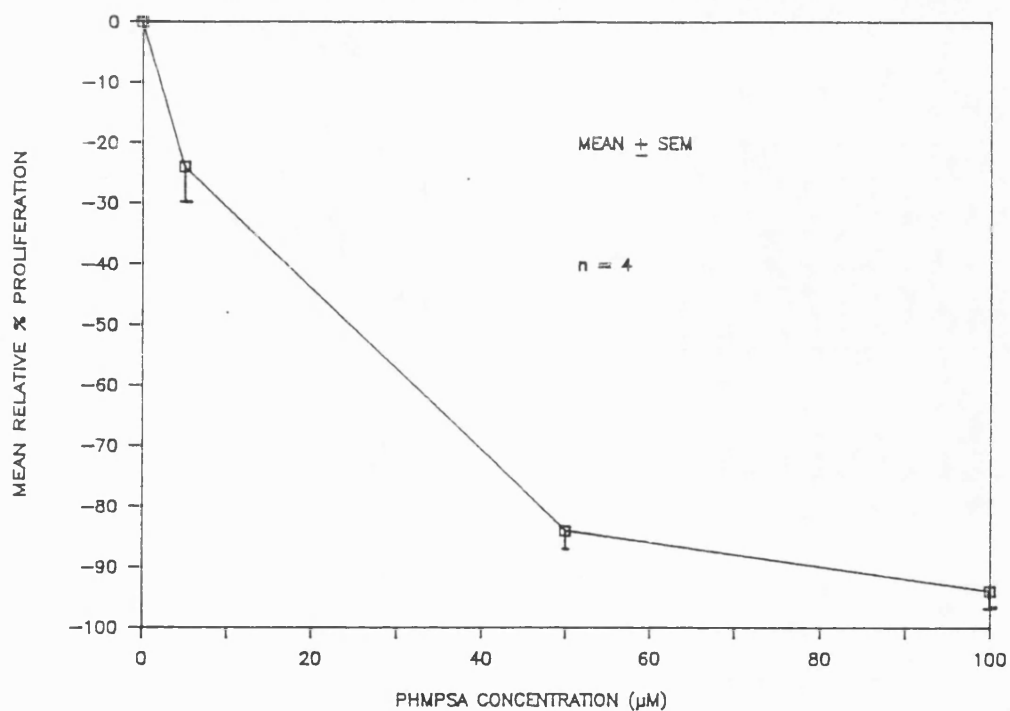


FIG. 4.69 EFFECT OF PRETREATMENT WITH PHMPA ON SUBSEQUENT PROLIFERATION
IN RESPONSE TO 1:200 PWM IN NORMAL SUBJECTS

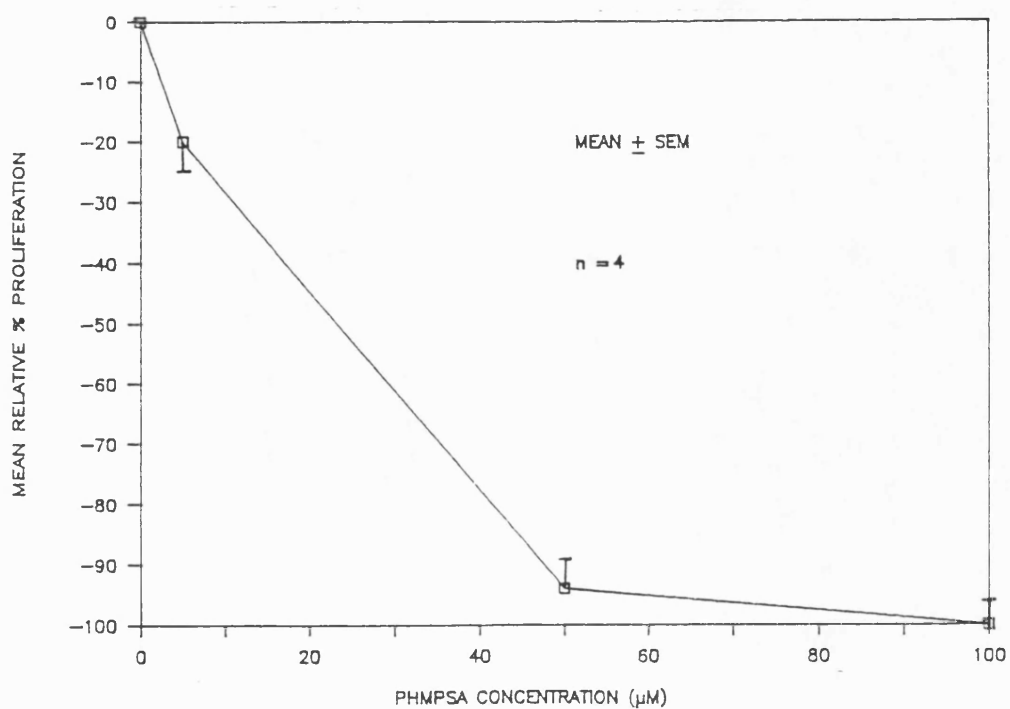
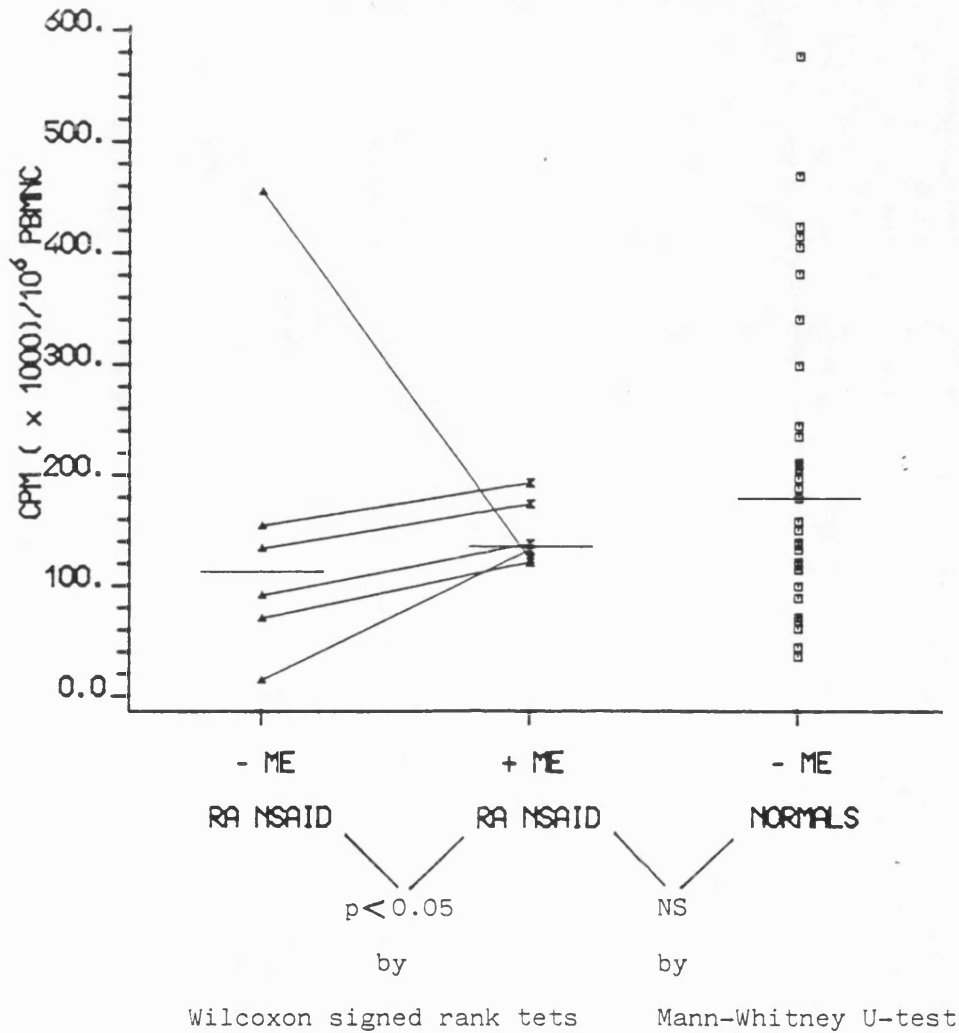


FIG. 4.70

EFFECT OF 2-ME ON CON-A INDUCED PROLIFERATION IN RA NSAID PATIENTS

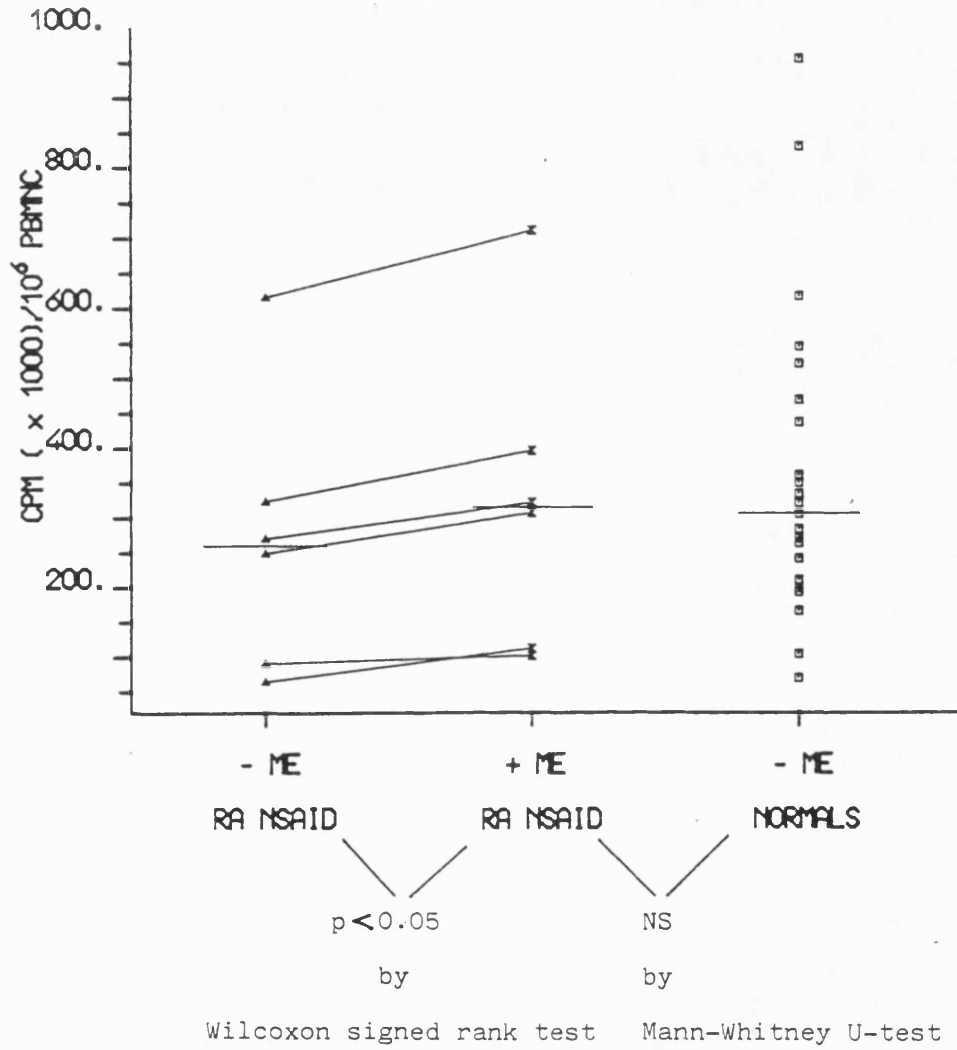


CPM were multiplied by 10 to give CPM/10⁶ cells.

Horizontal bars represent median CPM, having subtracted background counts.

FIG. 4.71

EFFECT OF 2-ME ON PHA STIMULATED PROLIFERATION IN RA NSAID PATIENTS

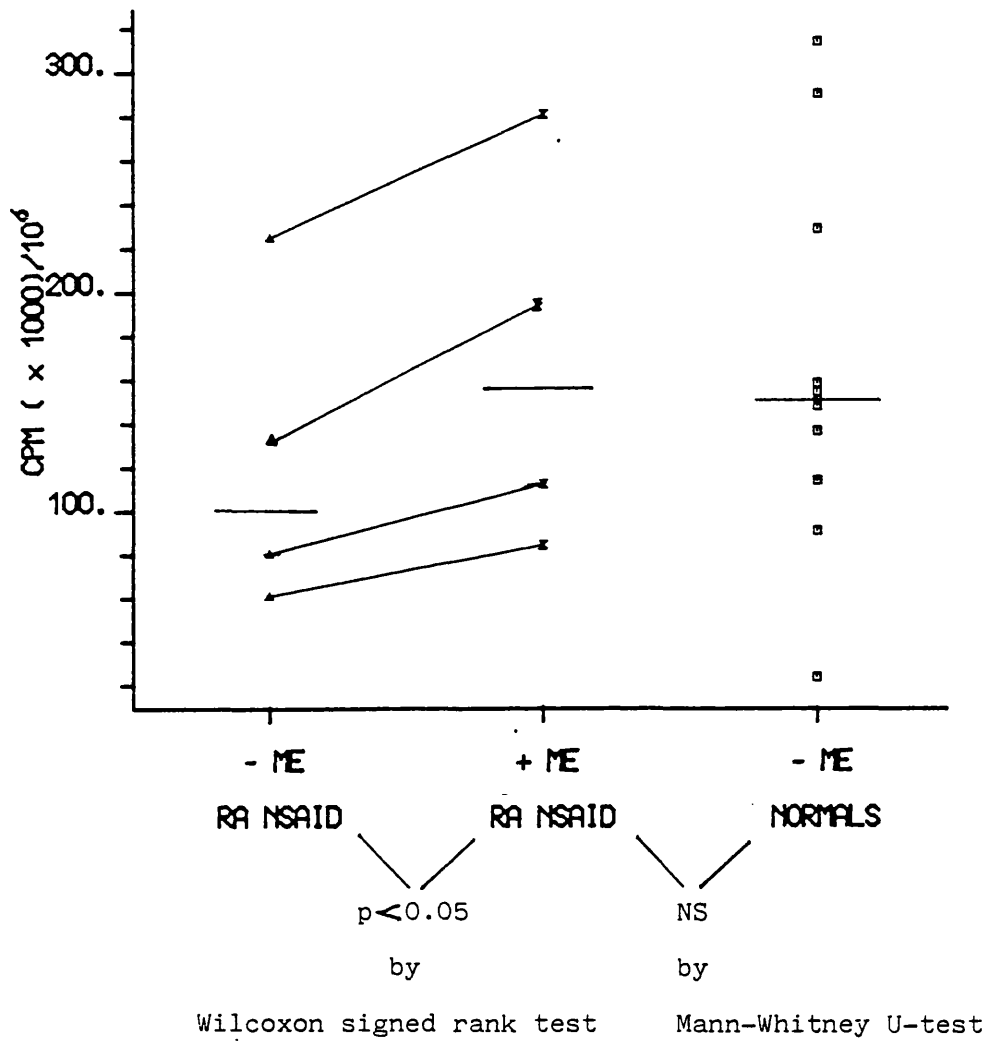


CPM were multiplied by 10 to give CPM/ 10^6 Cells.

Horizontal bars represent median CPM, having subtracted background counts.

FIG. 4.72

EFFECT OF 2-ME ON T3 Mab STIMULATED PROLIFERATION IN RA NSAID PATIENTS



CPM were multiplied by 10 to give CPM/10⁶ cells.

Horizontal bars represent median CPM, having subtracted background counts.

and in fact normalised the data when compared to proliferation in normal individuals. Since no differences were observed in patients with and without RF, all such patients were grouped together.

The defective proliferation observed in RA NSAID was found not to be due to different kinetics of proliferation, which was found to be the same in RA NSAID and normal individuals (data not shown), nor to RA NSAID patients showing a different dose response curve since this was similar in the two groups (Figs. 4.73-4.75). However, prior activation of the cells in vivo could account for the defective proliferation, since significantly raised background [^3H]-TdR uptake was found compared to normals (data not shown).

These results further illustrate that functionally important thiol groups are oxidized in RA NSAID and may play a role in the immunodysregulation observed.

4.18. EFFECT OF H_2O_2 ON MITOGEN STIMULATED PROLIFERATION.

Again it was considered advisable to see whether leaving the physiological oxidizing agent H_2O_2 in the 72 hours culture period with/without mitogens (Fig. 4.76-4.78) or pretreatment of cells with H_2O_2 and washing it off prior to mitogen stimulation (Figs. 4.79-4.80), paralleled results observed with PHMPA.

In both cases the same trend was observed, i.e. increasing doses of H_2O_2 increased suppression of proliferation. It was considered that experiments where H_2O_2 was left in the culture typified the in vivo situation in RA NSAID more closely than pretreatment experiments.

FIG.4.73 DOSE-RESPONSE CURVE FOR CON-A INDUCED PROLIFERATION
IN RA NSAID PATIENTS AFTER A 72 HOUR CULTURE PERIOD

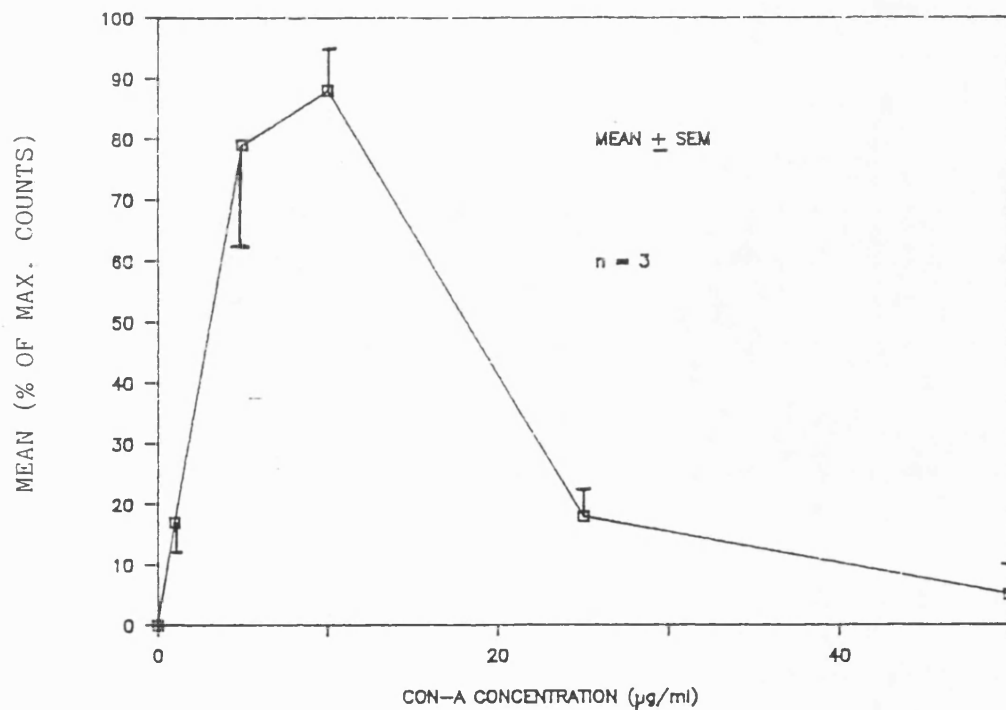


FIG.4.74 DOSE-RESPONSE CURVE FOR PHA INDUCED PROLIFERATION
IN RA NSAID PATIENTS AFTER A 72 HOUR CULTURE PERIOD

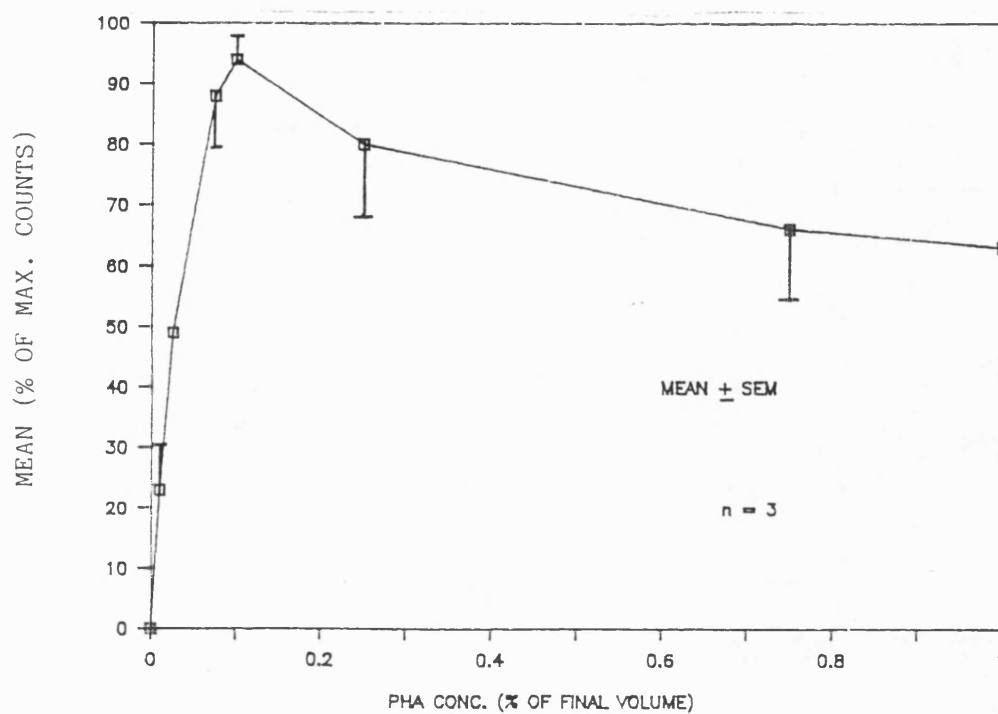


FIG. 4.75 DOSE-RESPONSE CURVE FOR Mab-T3 INDUCED PROLIFERATION
IN RA NSAID PATIENTS AFTER A 72 HOUR CULTURE PERIOD

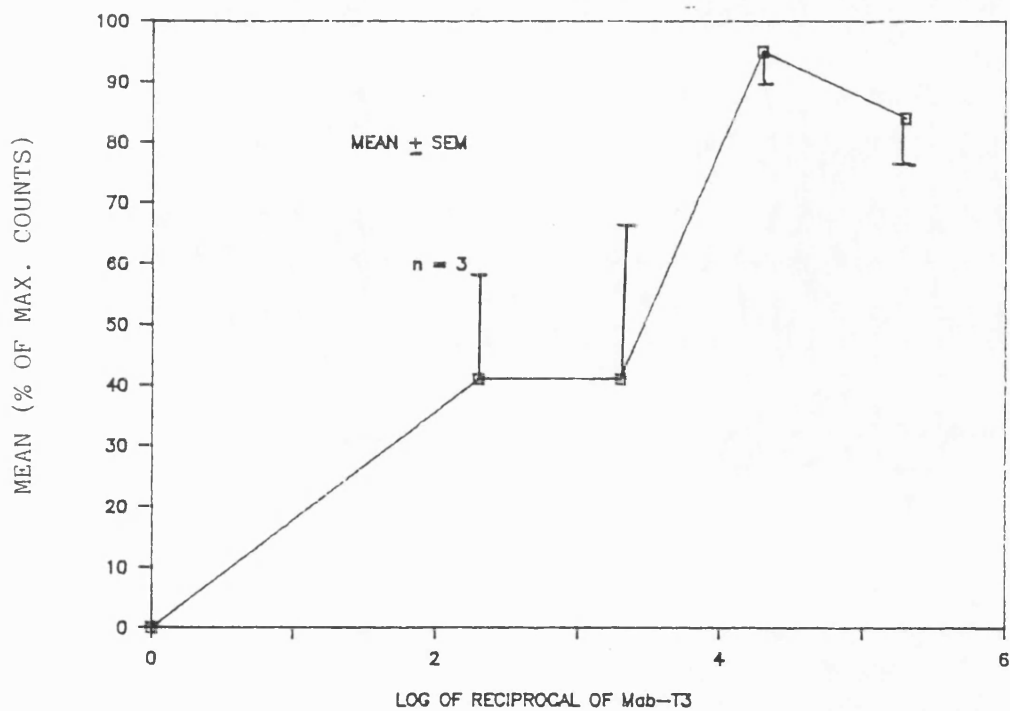


FIG. 4.76 EFFECT OF LEAVING H_2O_2 WITH 10 $\mu g/ml$ CON-A IN A 72 HOUR CULTURE ON SUBSEQUENT PROLIFERATION IN NORMAL SUBJECTS

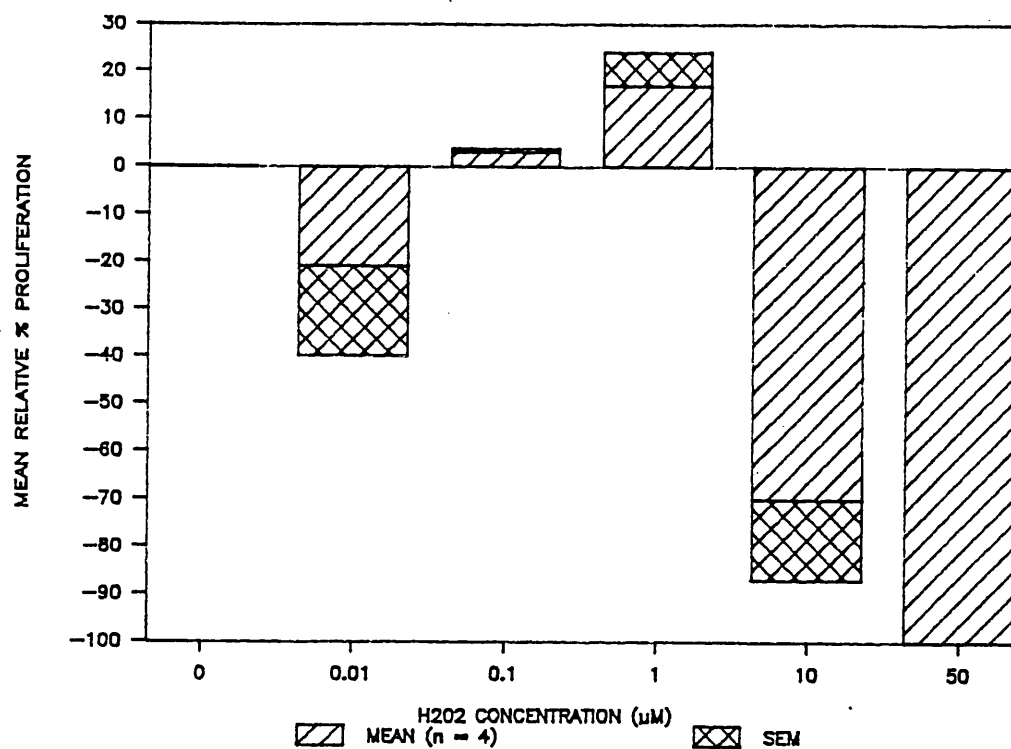


FIG. 4.77 EFFECT OF LEAVING H_2O_2 WITH 0.1% PHA V:V IN A 72 HOUR CULTURE ON SUBSEQUENT PROLIFERATION IN NORMAL SUBJECTS

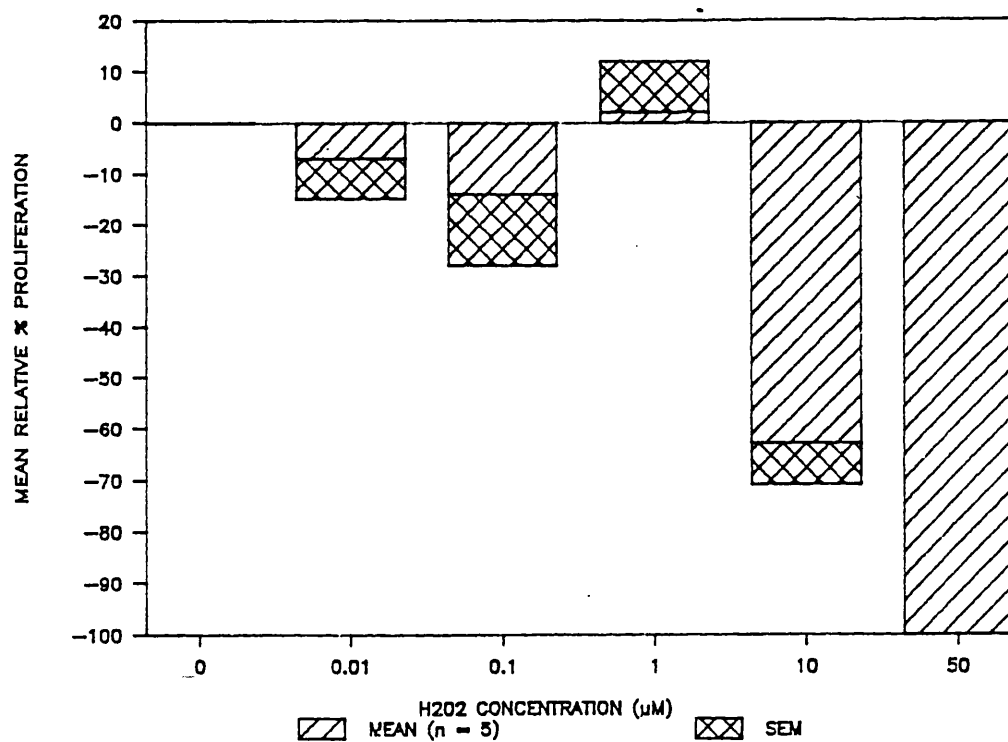


FIG.4.78 EFFECT OF LEAVING H_2O_2 WITH 1:20,000 Mab-T3 IN A 72 HOUR CULTURE ON SUBSEQUENT PROLIFERATION IN NORMAL SUBJECTS

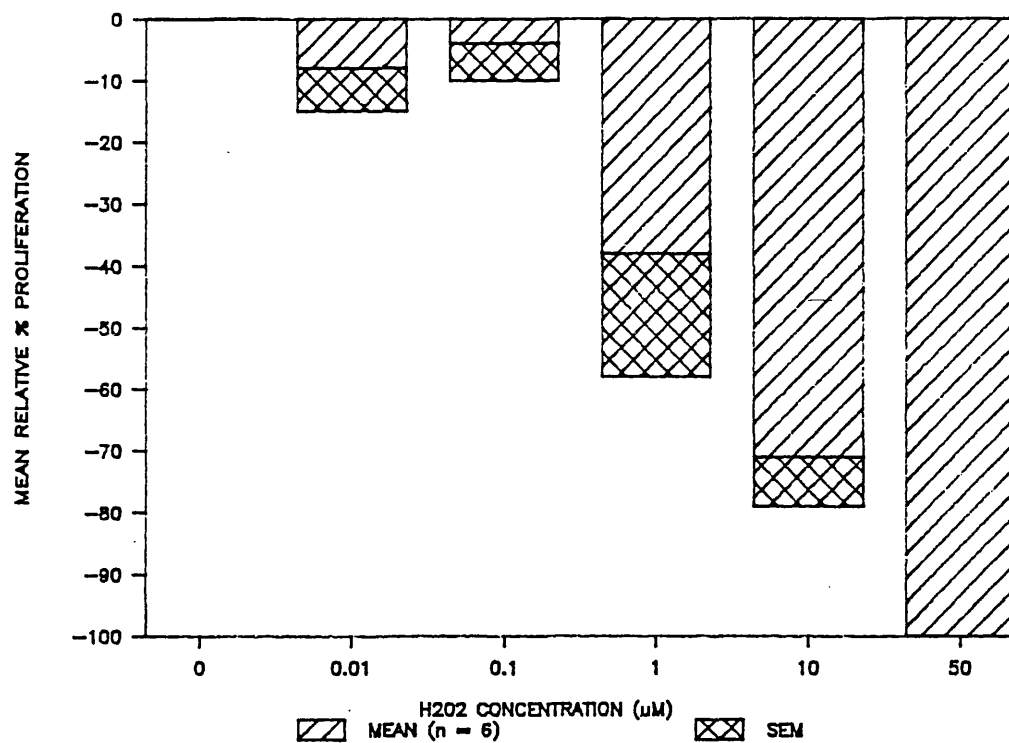


FIG. 4.79 EFFECT OF H_2O_2 PRETREATMENT ON SUBSEQUENT PROLIFERATION
IN RESPONSE TO 10 $\mu\text{g/ml}$ CON-A IN NORMAL SUBJECTS

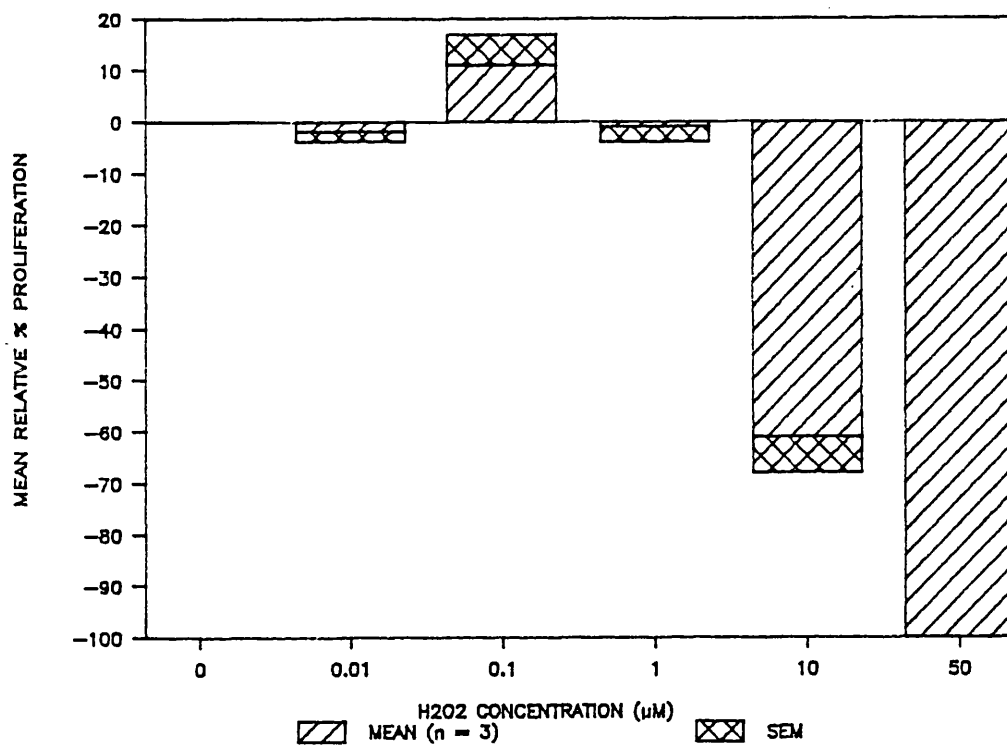
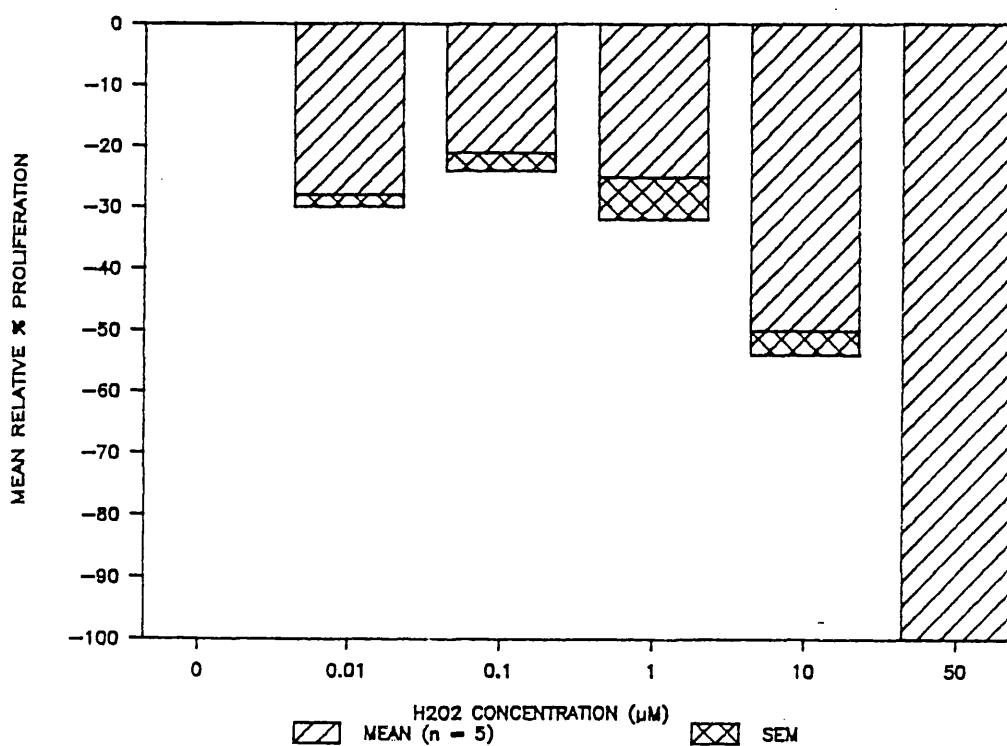


FIG. 4.80 EFFECT OF H_2O_2 PRETREATMENT ON SUBSEQUENT PROLIFERATION
IN RESPONSE TO 0.1% PHA V:V IN NORMAL SUBJECTS



Greater suppression of proliferation was observed when H_2O_2 was left in the culture than if it was washed off, probably because pretreatment enables cells to recover and re-reduce the oxidized cell surface groups to some extent. Inhibition of proliferation was also clearly visible since the cells were not spreading out, but remained tightly packed as a pellet at the bottom of the well.

Viabilities studies showed very little inhibition of viability in the range 10 nM to 1 μ M with a 50% reduction in cell viability at 10 μ M and 100% cell death 50 μ M onwards, both when cells were pretreated with H_2O_2 and when this was washed off, or when H_2O_2 was left in the culture +/- mitogen (Tables 4.29-4.30).

It is thought that physiologically local concentrations of between 10 nM and 10 μ M would certainly be possible in RA NSAID patients in sites of active inflammation and within this range the proliferative response to anti-CD3 Mab (Fig. 4.78) seems to be more sensitive to H_2O_2 than CON-A and PHA, probably because proliferation in response to anti-CD3 Mab requires even more cooperation between lymphocytes and monocytes (anti-CD3 responses involve FC receptor (FCR1) interaction on monocytes [13]), and both lymphocytes and monocytes have been shown to have cell surface thiol groups important for their interaction [1]. Possibly proliferation in response to CON-A and PHA are less monocyte dependent.

In the light of these and previous results, showing the thiol dependence of proliferation and CON-A induced suppressor activity, but essentially thiol independence of induction of CON-A induced

TABLE 4.29

PERCENTAGE OF VIABLE CELLS REMAINING AFTER TREATMENT FOR 1 HOUR WITH VARIOUS H_2O_2 CONCENTRATION, THEN WASHED OFF ($n = 4$).

$[\text{H}_2\text{O}_2]$	VIABILITY
0	99 %
10 nM	99 %
100 nM	98 %
1 μM	96 %
10 μM	50 %
100 μM	0 %
1 mM	0 %
10 mM	0 %

TABLE 4.30

VIABILITY STUDIES IN THE PRESENCE OF H_2O_2 LEFT IN CULTURE FOR 72 HOURS WITH/WITHOUT MITOGEN IN TWO NORMAL SUBJECTS. RESULTS ARE EXPRESSED AS \bar{x} % VIABLE CELLS ($n = 2$).

$[\text{H}_2\text{O}_2]$	NO MITOGEN	10 $\mu\text{g}/\text{ml}$ CON-A	0.1% V/V PHA
0 nM	99%	96%	96%
10 nM	99%	95%	96%
20 nM	97%	95%	95%
100 nM	95%	95%	95%
200 nM	95%	95%	95%
1 μM	95%	95%	95%
2 μM	95%	95%	95%
10 μM	48%	69%	50%
50 μM	0%	0%	0%
100 μM	0%	0%	0%

suppressor activity, it was wondered what aspects could be thiol dependent. Since all 4 mitogens were inhibited to the same extent at the same concentration of PHMPA, it was thought that perhaps a similar mechanism was responsible for inhibition in each case.

Three possibilities were investigated, since they also bore relevance to the CON-A induced suppressor assay: because it has been suggested that the CON-A induced suppressor assay is an artefact i.e. it has been suggested that suppressor cells bear IL-2 receptors which are able to bind IL-2 made by T-cells in the responder population in the coculture and thus reduce its concentration for use by the IL-2R bearing CD4⁺ and B-cells in the responder population, which are thus artefactually inhibited from producing immunoglobulins by defective IL-2 levels in the medium [14-16].

Thus, the thiol dependence of IL-2 production, IL-2 receptor induction, IL-2 receptor expression and ability to bind IL-2 were analysed, although it must be remembered that other interleukins and their respective receptors and other soluble immunoregulatory factors could equally or alternatively be involved, or some other mechanism altogether.

4.19. PRODUCTION OF "EDUCATED" CELLS FOR IL-2 ESTIMATION.

First attention was drawn to the idea that perhaps IL-2 production could be inhibited by PHMPA pretreatment of normal PBMNC, so an IL-2 assay was set up to investigate this.

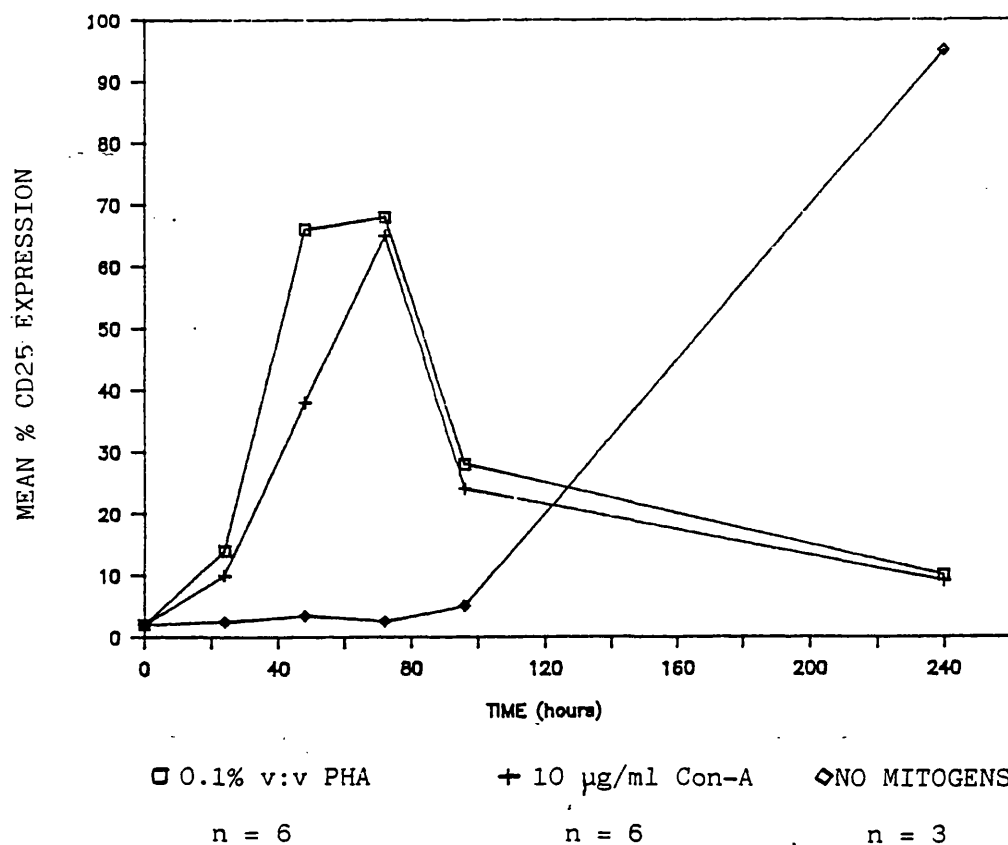
The IL-2 assay consisted of a system in which cells were "educated" with RPMI containing 10% FCS for 10 days, after which the IL-2 receptor is expressed as shown in fig. 4.81 and the cells can subsequently proliferate when rIL-2 or IL-2 containing supernatants are added [17].

It was observed that different batches of FCS had different abilities to "educate" PBMNC to become capable of proliferating in response to rIL-2, as shown in fig. 4.82. Some batches are so potent that they even cause IL-2 production as well as IL-2R expression, resulting in spontaneous proliferation of "educated" cells. Such "mitogenic" batches were avoided for the assay in this project, since they could create problems when dealing with very low IL-2 levels expected in mitogen stimulated PBMNC supernatants, because the difference between background proliferation and proliferation as a result of supernatant addition could be minimal, thus reducing the reliability of the results obtained.

It was also observed that in the range 0.0001-10 IU/ml (a range in which the high affinity IL-2 receptor would be in operation), using a single batch of FCS, individuals varied considerably in the proliferative capacity in response to rIL-2, as shown in fig. 4.83

Considerable individual variation in proliferative capacity using a single batch of FCS was also observed when the dose range of rIL-2 was increased to 0.01-1,000 IU/ml (to cover a range in which the high affinity and intermediate affinity IL-2 receptors would be in operation, 0-100 IU/ml and 100- > 1000 IU/ml respectively, Fig.4.84).

FIG. 4.81 CD-25 EXPRESSION INDUCED BY MITOGENS (CON-A OR PHA)
OR BY FOETAL CALF SERUM ALONE



Showing peak CD-25 expression occurs at 72 hours after mitogen stimulation
but only after 240 hours in the absence of mitogens.
All culture media contain 10% foetal calf serum.

FIG.4.82 EFFECT OF DIFFERENT BATCHES OF FCS ON ABILITY TO INDUCE

"EDUCATED CELLS" FROM PBMNC AFTER A 10 DAY CULTURE PERIOD -

AS SHOWN BY THEIR SUBSEQUENT PROLIFERATION TO rIL-2 10-0.0001 IU/ml

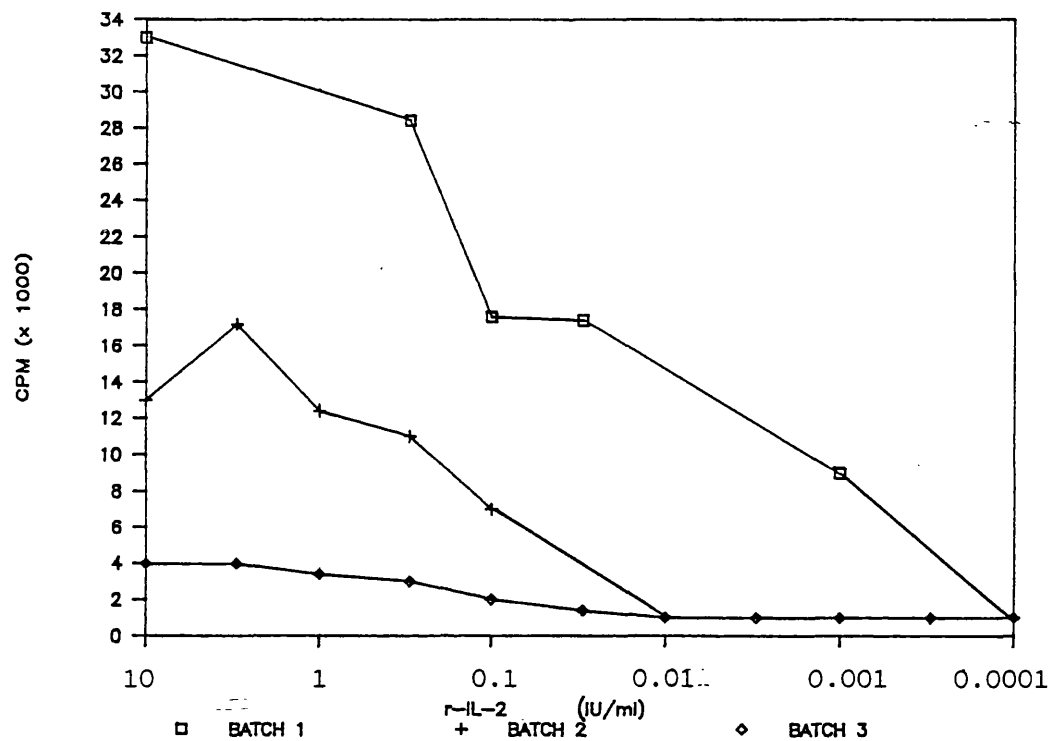


FIG. 4.83 VARIATION IN SENSITIVITY OF EDUCATED CELLS FROM DIFFERENT NORMAL SUBJECTS TO RESPONDE TO rIL-2 10 - 0.0001 IU/ml

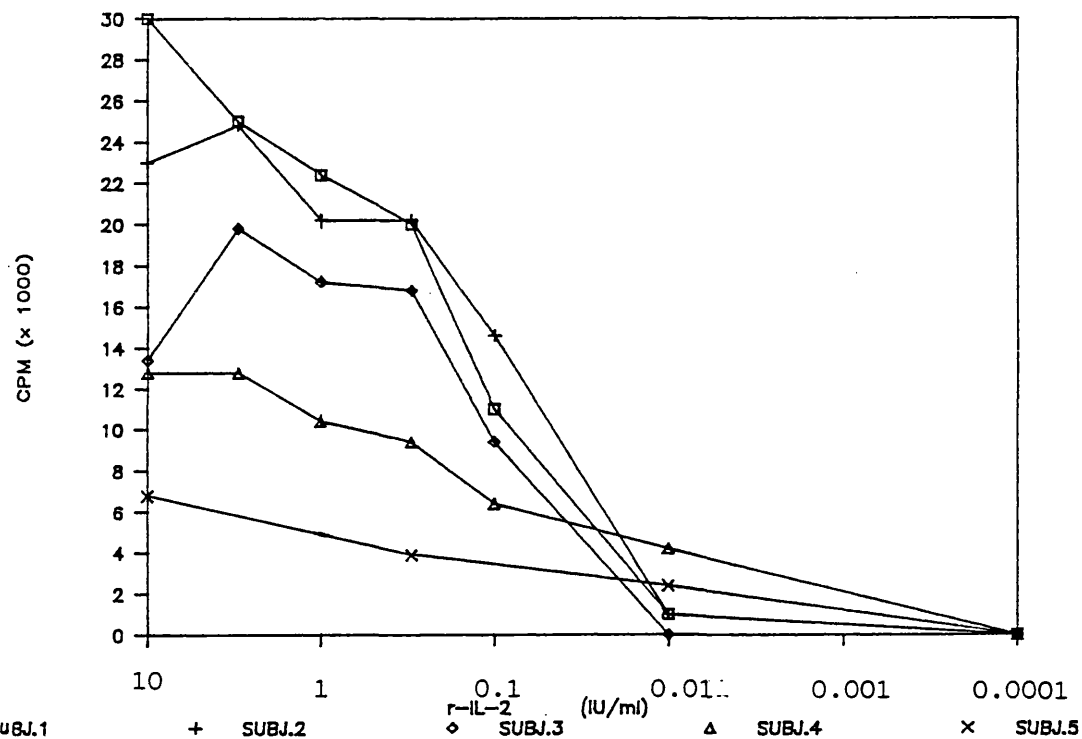
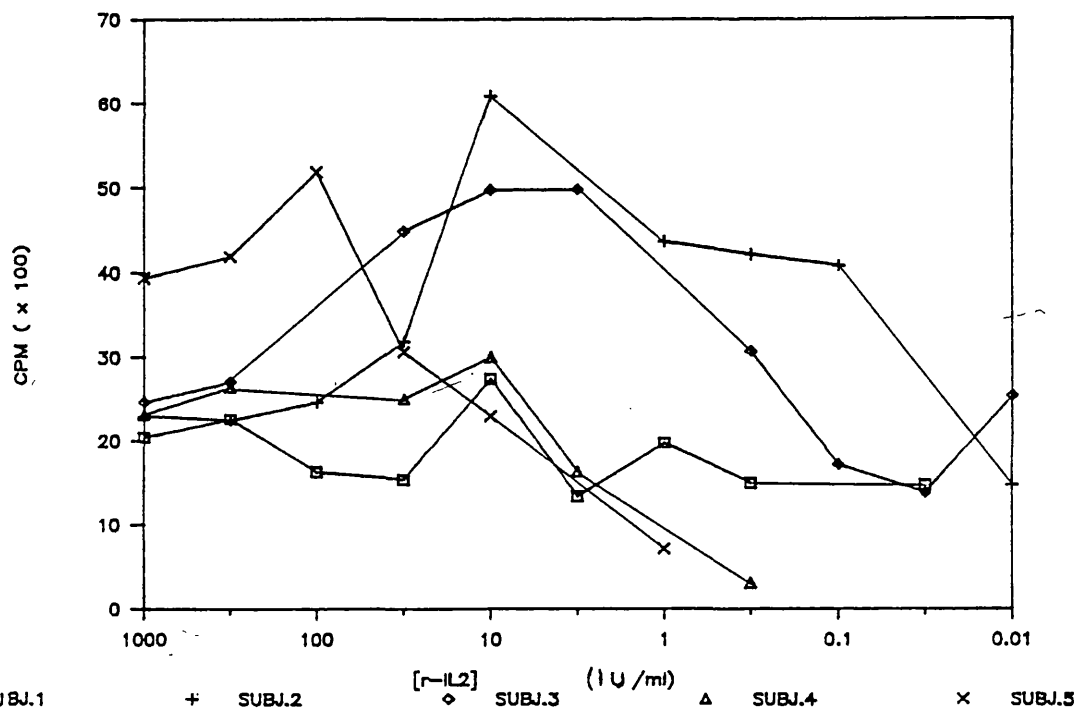


FIG. 4.84 RESPONSE OF EDUCATED CELLS FROM 5 NORMAL SUBJECTS TO rIL-2 IN RANGE FROM 1000 TO 0.01 IU/ml



It was shown that "educated" cells were sensitive to CON-A (Fig. 4.85) and PHA (data not shown), so it was important to have a washing step during IL-2 supernatant production to rid the supernatant of CON-A or PHA which would otherwise interact with "educated" cells and falsely raise their counts.

To avoid the washing step mitogens can be removed by binding to columns e.g. PHA can be removed by passing the sample through a CON-A sepharose 4B column, but for the small 1 ml volumes used in this experiment, this method would not have been economical and it was found as long as efficient washing was carried out, the mitogens did not interfere in the subsequent IL-2 assay. Alternatively, other methods for removing lectins from supernatants are: by HPL chromatography, adsorption to chicken red cells, absorption to silicic acid or sequential NH_4SO_4 precipitation.

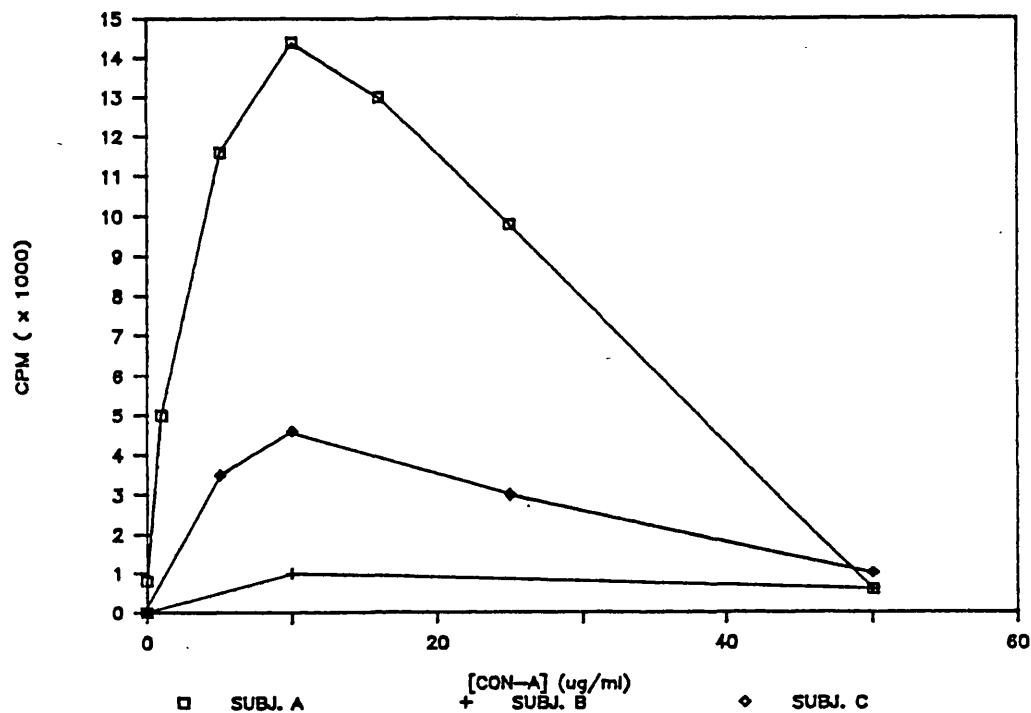
Experiments were attempted to see whether addition of 3 mM, 30 mM or 0.3 M α -methylmannoside to the "educated" cell assay for IL-2 measurements would allow the use of CON-A stimulated IL-2 containing supernatants to be used directly without requiring a washing step and without interfering with the ability of "educated" cells to respond to rIL-2. Initial experiments showed this to be a possibility and if time had permitted this would have been the method of choice.

4.20. EFFECT OF PHMPA ON IL-2 PRODUCTION.

Initially experiments were carried out to find the optimum dose of CON-A for IL-2 production, and the kinetics of IL-2 production to

FIG. 4.85

SENSITIVITY OF EDUCATED CELLS TO CON-A



Since educated cells proliferate in response to Con-A to varying degrees in different subjects, these results indicate the need to eliminate Con-A from the cell supernatant before measuring IL-2.

find at what point supernatants should be harvested such that maximum IL-2 concentrations would be observed. The former was found to be 10 $\mu\text{g/ml}$ CON-A (Fig. 4.86) and it was found that maximum IL-2 output occurred between 24 and 48 hours in culture and tailed off either side of this (Fig. 4.87).

It was found that pretreatment of normal PBMNC with 50 μM PHMPSA (Fig. 4.88) did not significantly affect their subsequent ability to produce IL-2, nor did 50 μM PHMPSA inhibit the ability of already CON-A stimulated cells to secrete IL-2 (data not shown).

Similar results were obtained using suboptimal and superoptimal concentrations of CON-A and PHA and using 0.1% v/v PHA instead of CON-A, found to be an optimum dose for stimulation of IL-2 production with PHA (data not shown).

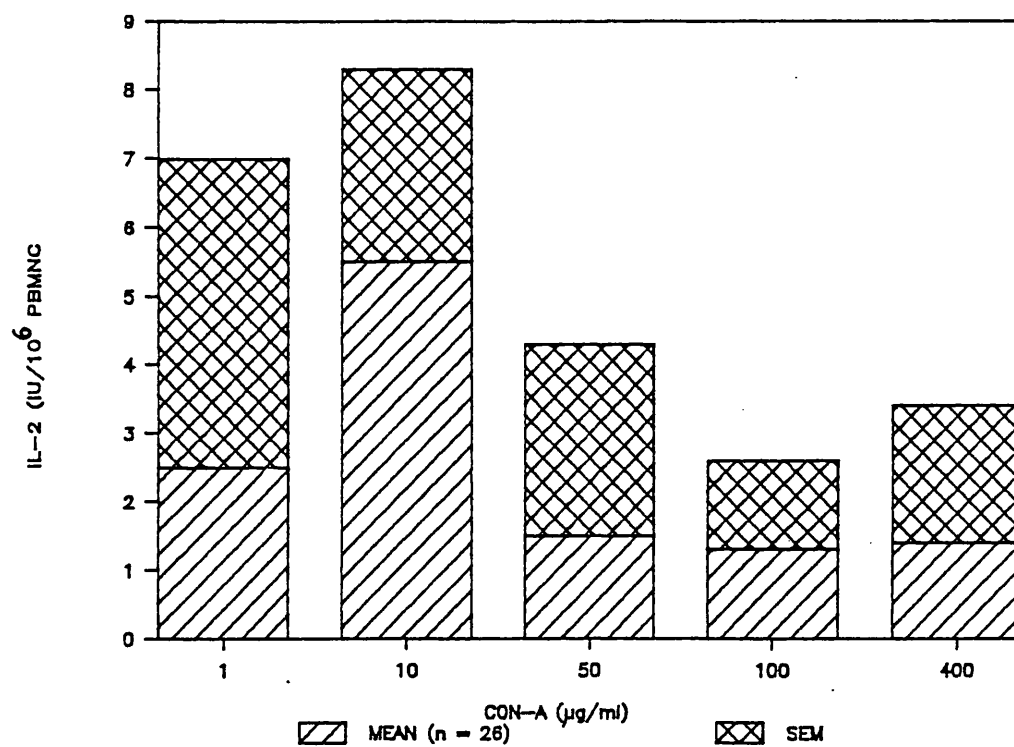
Parallel studies using the physiological thiol oxidant H_2O_2 left in the culture also showed no effect on IL-2 production (data not shown). Thus inhibition of mitogen stimulated proliferation by PHMPSA is not a result of inhibition of IL-2 production.

4.21. COMPARISON OF IL-2 PRODUCTION IN VARIOUS PATIENT GROUPS AND NORMALS.

Since various workers suggested IL-2 is required for maturation and activity of suppressor cells [18], it was wondered whether defective CON-A induced suppressor activity in RA NSAID was due to defective IL-2 production, a likely hypothesis in the light of reduced levels of $\text{T}_{\text{S/I}}$, "naive" cells found by some workers in the blood of RA

FIG. 4.86

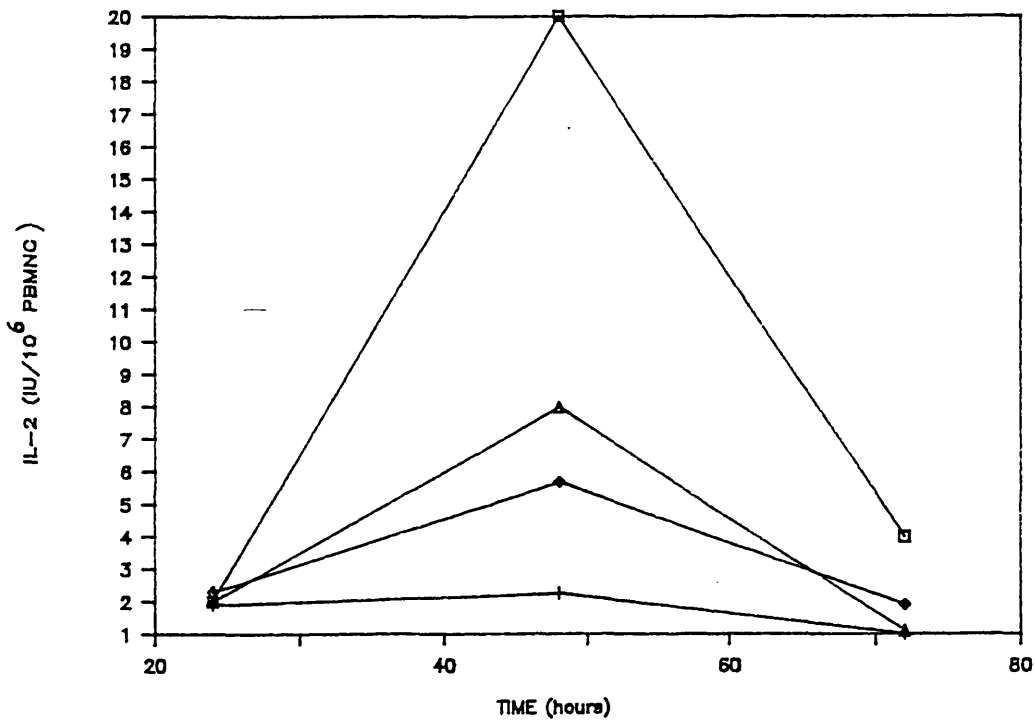
IL-2 STANDARD CURVES



Showing that 10 μg/ml Con-A is optimal for maximal IL-2 production in 1 ml cultures containing 10⁶ PBMNC.

FIG. 4.87

KINETICS OF IL-2 PRODUCTION IN NORMALS



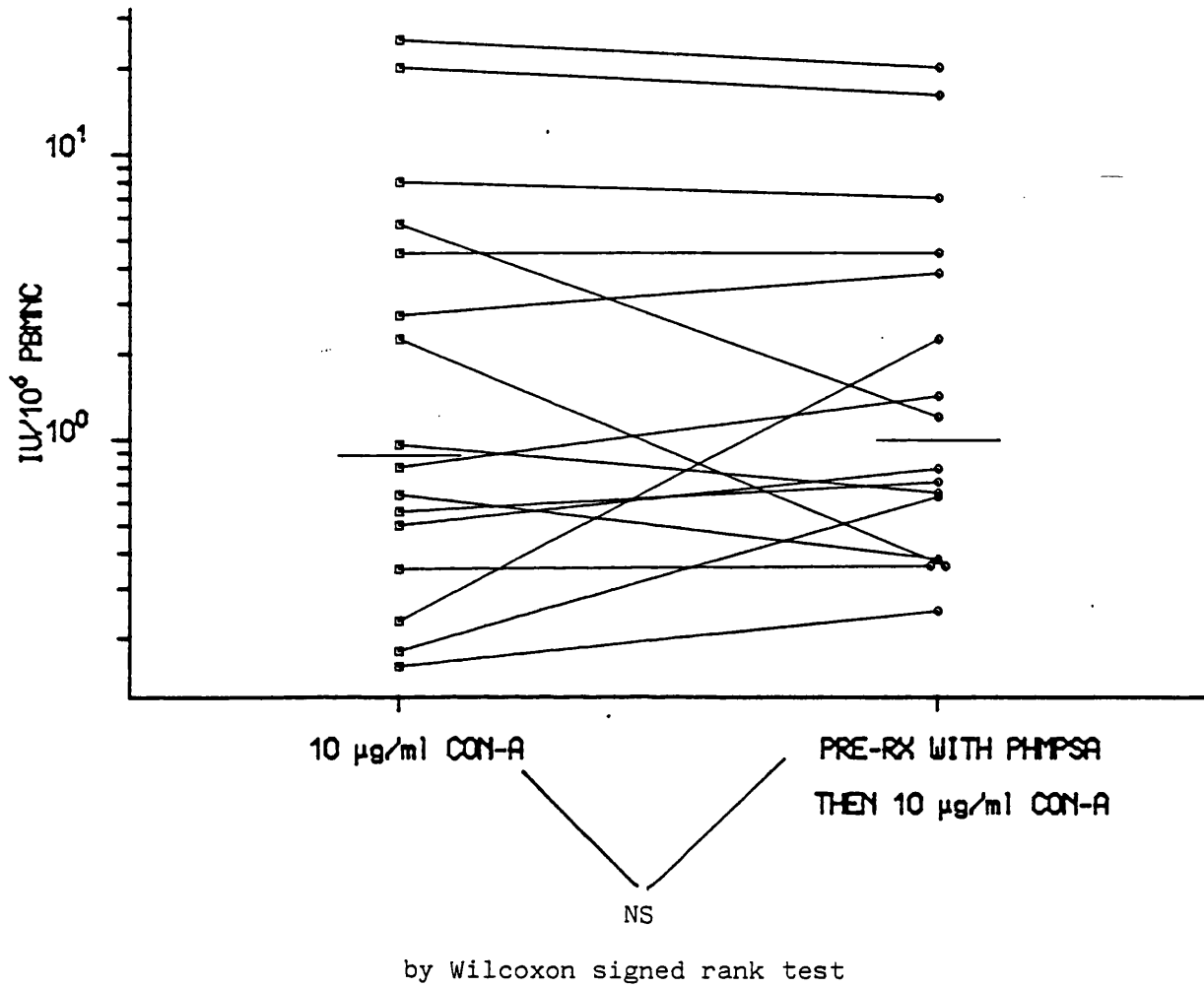
Time indicates total time in culture, therefore:

- 12 hours with 10 µg/ml Con-A, wash, 12 hrs. mitogen-free = 24 hour culture
- 24 hours with 10 µg/ml Con-A, wash, 24 hrs mitogen-free = 48 hour culture
- 24 hours with 10 µg/ml Con-A, wash, 48 hrs mitogen-free = 72 hour culture

Showing a total culture time of 48 hours is optimal for maximal IL-2 concentrations to be obtainable in normal subjects.

FIG. 4.88

EFFECT OF PRE-TREATMENT WITH 50 μ M PHMPA
ON ABILITY OF CON-A TO INDUCE IL-2 PRODUCTION



Horizontal bars represent median IL-2 concentration.

NSAID [19], thought at one time to be the main producer of IL-2 [20].

Fig. 4.89 shows no significant differences in IL-2 production in total RA NSAID patients (\pm EAD, \pm RF) compared to normals, nor if separated into RA NSAID -EAD or +EAD (i.e. with more severe disease). No difference between -EAD and +EAD RA NSAID was also found. AS patients also showed no significant difference in IL-2 production compared to normals. There was considerable individual variation in IL-2 output: this observation has been noted by other workers [21]; hence the need to show the data on a log scale. The range of IL-2 concentrations found here are similar to those quoted by others [21]. In no groups was there spontaneous release of IL-2. In the light of the above statement not surprisingly experiments carried out to see the effect of 2-ME on IL-2 production in total RA NSAID (+/-EAD, +/-RF) showed no significant differences compared to normals (Fig 4.90), nor if 2-ME was added to cells from either -EAD or +EAD patients (data not shown).

Although the RA NSAID patients were shown to secrete levels of IL-2 not significantly different to that of normals, there was a tendency to show increased deficiency in IL-2 production in those RA NSAID patients showing more severe disease, that is +EAD, and in fact 1 out of the 11 of these patients showed the lowest IL-2 production of all subjects investigated. It was wondered whether this tendency was because the kinetics of IL-2 production was different to that in normals; however fig. 4.91 shows that it is not the case.

Since it has been suggested that IL-2 is important in the

FIG. 4.89

IL-2 PRODUCTION IN NORMALS, RA NSAID, AS PATIENTS AFTER STIMULATION WITH 10 μ g/ml CON-A

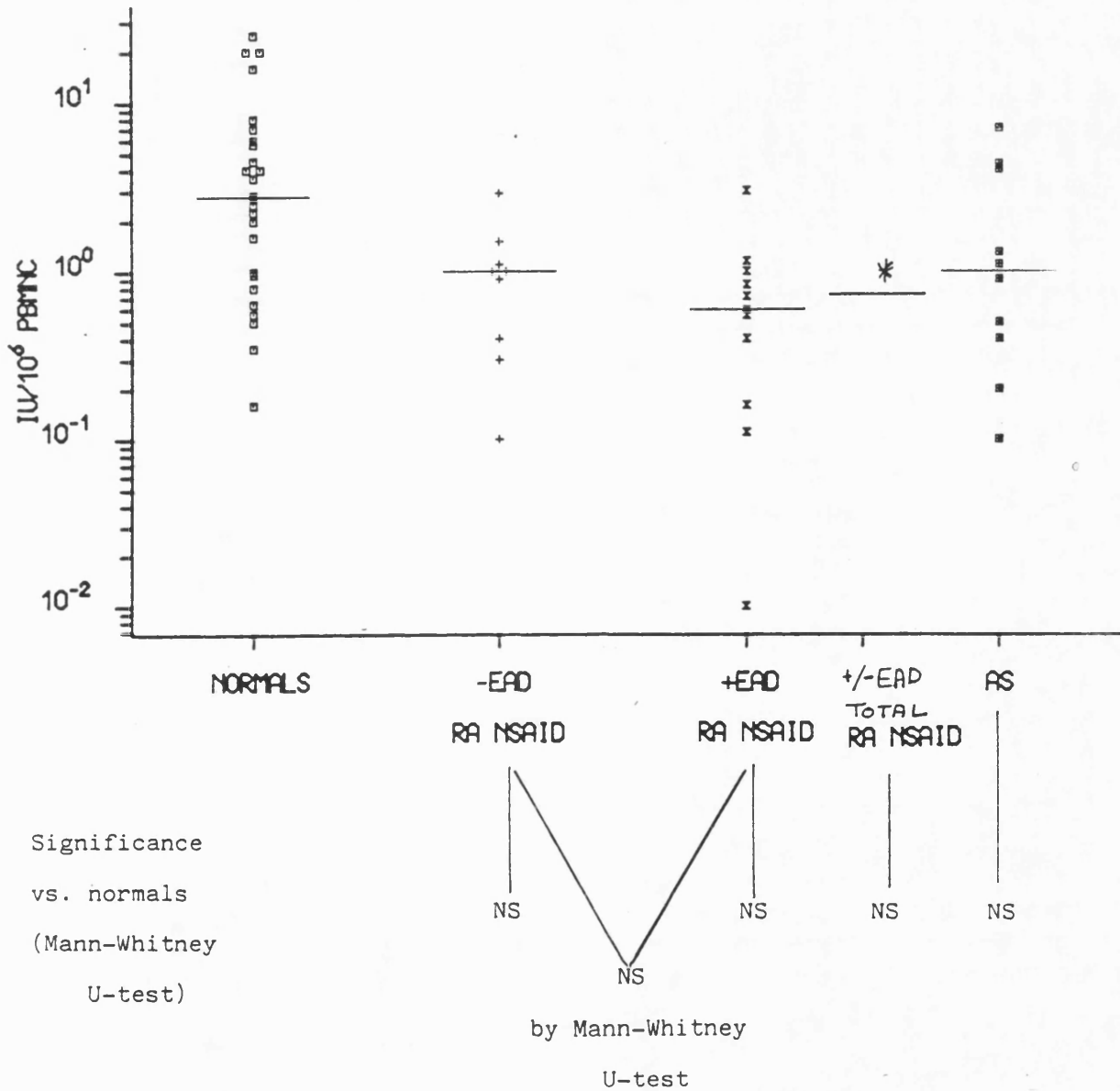
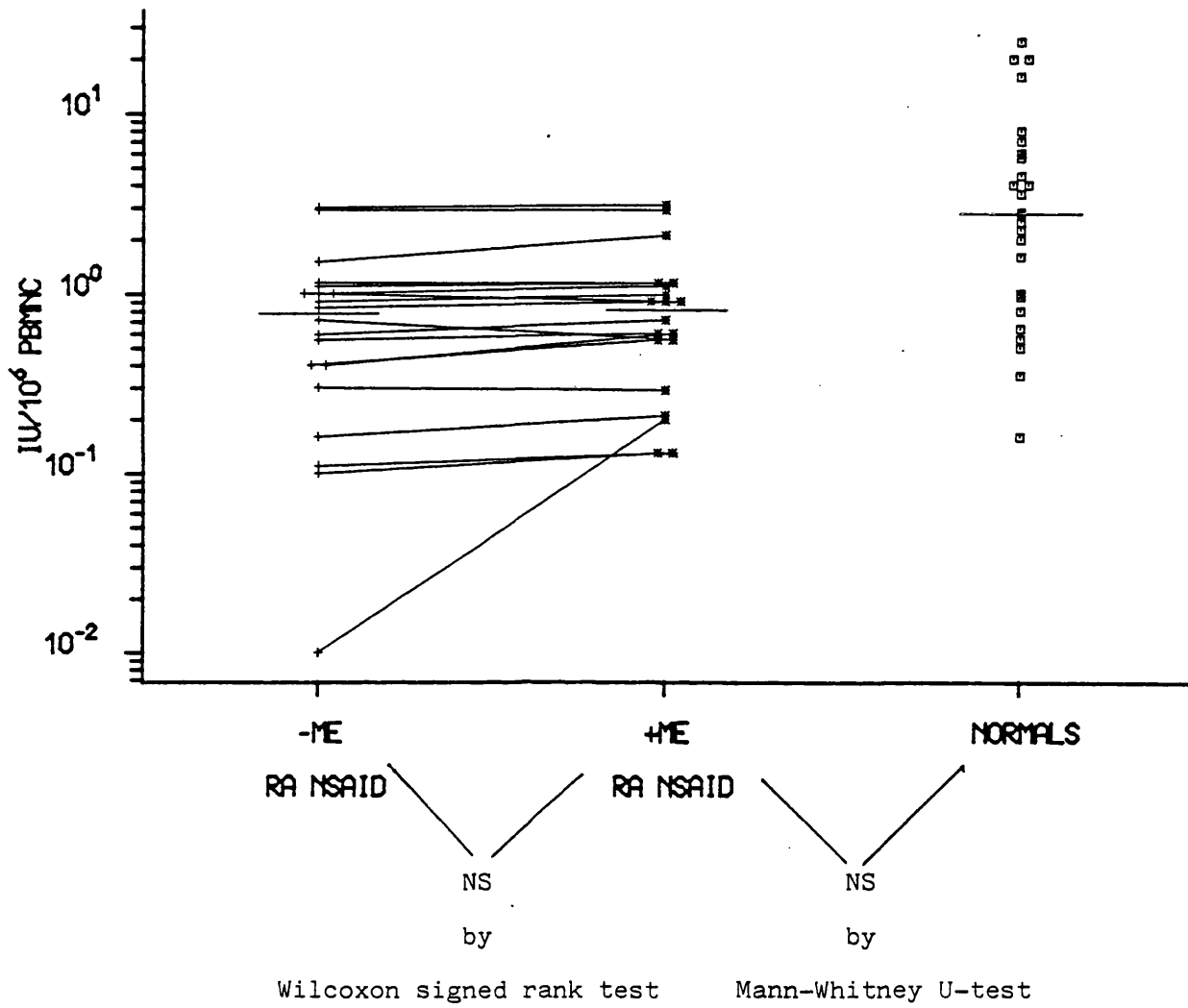


FIG. 4.90

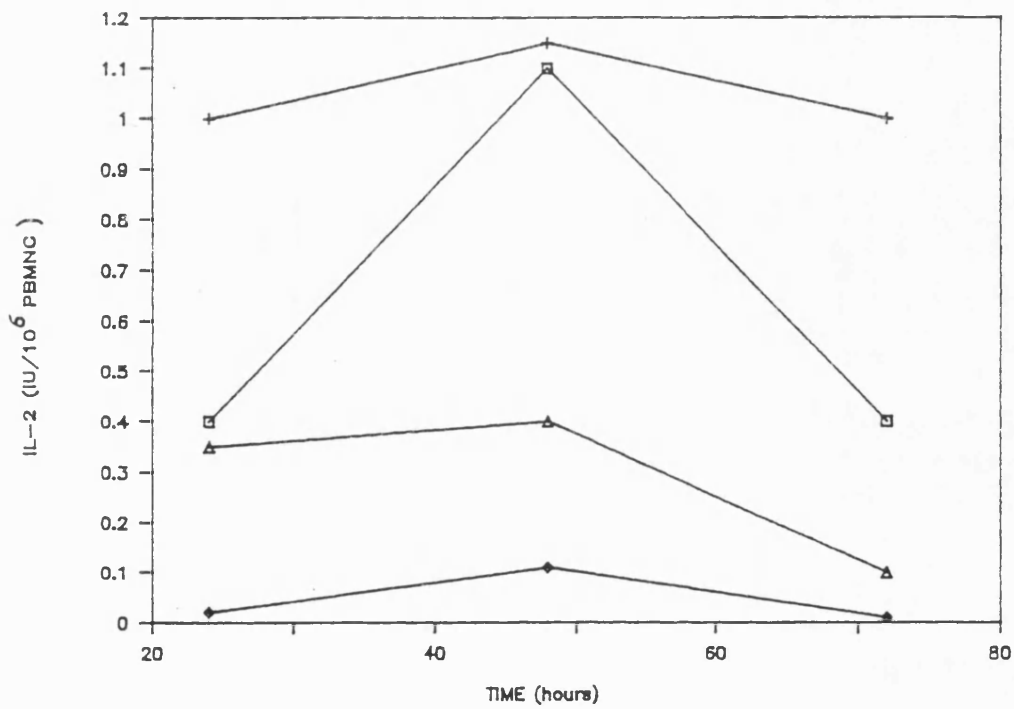
EFFECT OF 2-MERCAPTOETHANOL ON IL-2 PRODUCTION
IN RA NSAID PATIENTS



Horizontal bars represent median IL-2 concentration.

FIG. 4.91

KINETICS OF IL-2 PRODUCTION IN RA NSAID



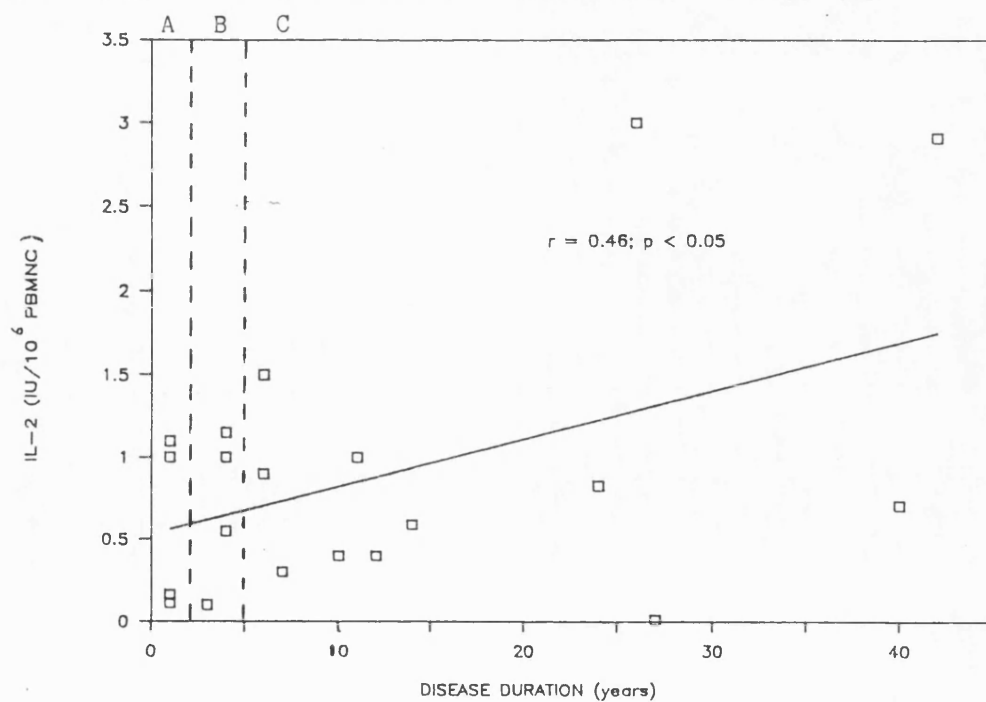
Showing a total culture time of 48 hours is optimal for maximal IL-2 concentrations to be obtainable in RA NSAID patients.

maturation and expression of PWM induced suppressor activity [18] and possibly CON-A induced suppressor activity, in view of the appearance of CD25 on CON-A activated suppressor cells [22], it was wondered whether defective suppressor activity for IgG and IgM synthesis in RA NSAID correlated with the tendency for defective IL-2 production. Not even a trend towards correlation was observed between IL-2 and suppression of IgG synthesis ($n = 20$, $r = 0.17$); however a trend towards a negative correlation, although not significant, was observed between IL-2 and suppression of IgM synthesis ($n = 9$, $r = -0.42$), perhaps indicative of a link between defective suppressor activity and reduced IL-2 levels. There was no significant correlation between CON-A induced suppressor activity for IgG or IgM synthesis and IL-2 production in RA NSAID +EAD nor in RA NSAID -EAD (data not shown).

Fig. 4.92 shows that a significant correlation ($r = 0.5$, $p < 0.05$) was observed between IL-2 and disease duration in RA NSAID, suggesting that patients with "very early active disease", i.e. less than 2 years, and "early established disease", i.e. 2 to 5 years, may show worst IL-2 production than patients with "long-standing chronic active disease", i.e. more than 5 years. However, comparison of median IL-2 levels within these 3 arbitrary disease duration divisions showed no significant differences.

Correlations between IL-2 and clinical indices of disease activity (RF and EMS) were examined. Not even a trend towards a correlation was observed between IL-2 and RF, thus it was decided that in all graphs patients with and without RF would be grouped together. However, for IL-2 and EMS a trend towards a negative

FIGURE 4.92 CORRELATIONS BETWEEN IL-2 AND DD



Median (range) IL-2 production for the 3 disease duration divisions:

A = 0.6 (0.11 - 1.10)

B = 0.8 (0.10 - 1.15)

C = 0.8 (0.01 - 2.91)

correlation was observed, although not significant ($n = 6$, $r = -0.63$).

Correlations between IL-2 and clinical indices of the acute phase response (ESR, CRP, viscosity, WBC, PLTS) were examined, as were correlations between IL-2 and Hb, IL-2 and serum -SH, and IL-2 and proliferation. No significant correlations were observed, nor were any trends towards correlations visible. The data for IL-2 vs. serum thiols agreeing with the data showing a lack of effect of PHMPSA and H_2O_2 on IL-2 production. However, a significant correlation was observed between IL-2 and age ($r = 0.47$, $p < 0.05$), showing IL-2 levels rose with age (Fig. 4.93).

4.22. EFFECT OF PHMPSA ON CELL SURFACE ANTIGEN EXPRESSION.

Another possible explanation for the inhibitory effect of PHMPSA on proliferation could be that PHMPSA inhibits IL-2 receptor induction or its expression, or perhaps the expression of some other immunoregulatory important cell surface antigens, e.g CD3, CD2, CD4, CD8, CDW29 and CD45R.

At present only a Mab to the TAC antigen of the IL-2 receptor is available, so the following series of Mabs were utilised, their specificity being described in Table 3.1: SOTON T_3 , SOTON T_{11} , SOTON T_4 , SOTON T_8 , WR16, WR17, WR18, WR19, HNK-1 SOTON M2 and anti-TAC.

It was first important to standardise the fluorescent staining technique, to find the optimal dilution of the FITC-conjugate. A 1:20 dilution was found to be optimal (Fig. 4.94), for all Mabs used.

FIG. 4.93

IL2 VERSUS AGE IN NSAID PATIENTS

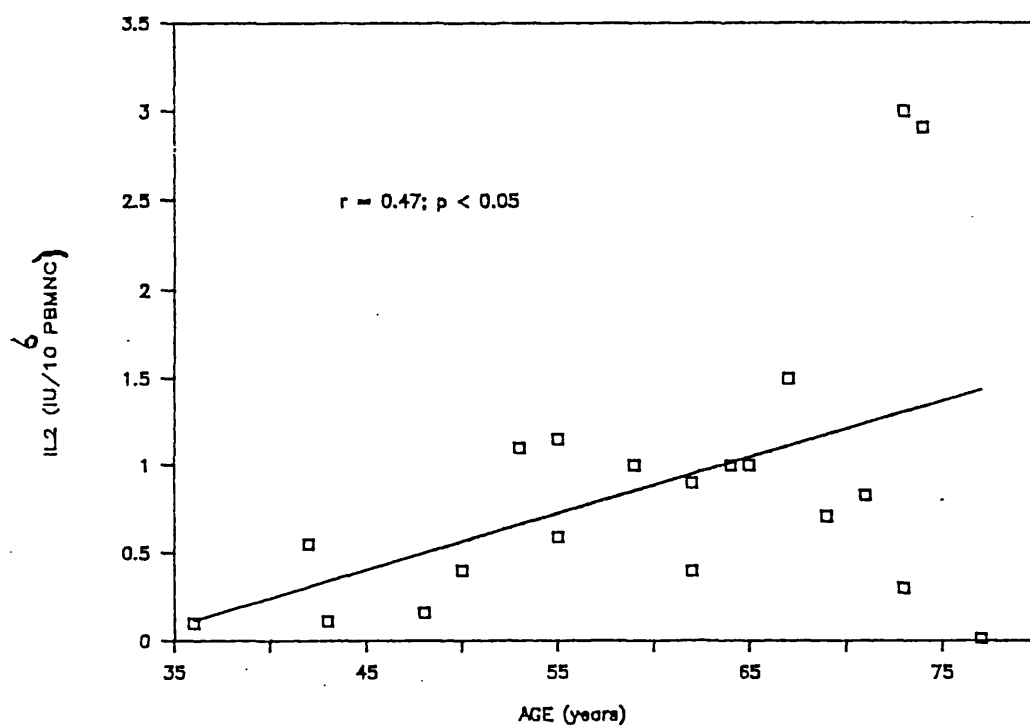
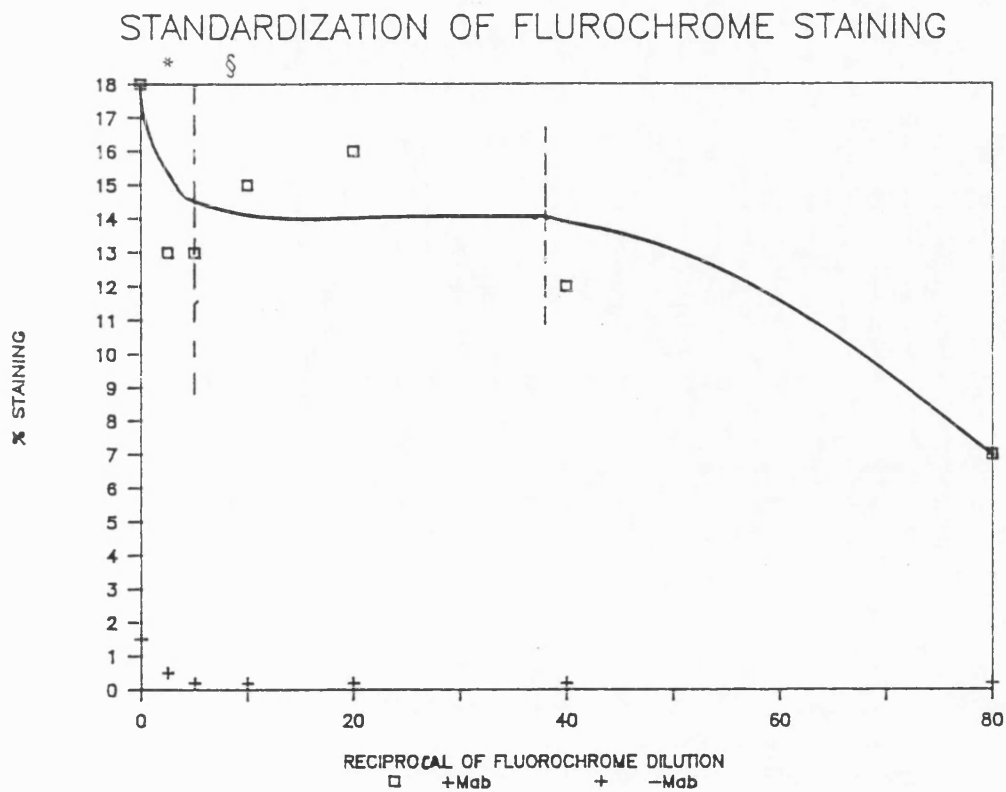


FIG. 4.94



* = PROZONE

§ = PLATEAU

Mab used was WR17. Points represent mean for $n = 2$ individuals.

Showing that 1:20 is an optimal dilution of FTIC conjugate at the midway point of the plateau region.

A 1:20 dilution was found to be optimal for use with all other Mabs.

Since the TAC antigen is only expressed on activated cells, e.g. on activated T-cells [22], B-cells [23], mast cells [24], CON-A induced suppressor cells, PWM-stimulated helper and suppressor cells and cytotoxic killer cells, but not their precursor cells [25], but is not expressed on resting T, B, MØ, mast cells, or memory T-cells, an optimal concentration of CON-A or PHA to induce TAC expression had to be found. Figs. 4.95-4.96 show 10 µg/ml CON-A and 0.1% v/v PHA respectively to be optimal; Figs. 4.97-4.99 show typical fluorescence observed using anti-TAC Mab.

The kinetics for optimum expression of the TAC antigen had to be found and fig. 4.81 shows that a 72 hours culture is optimal, levels falling dramatically either sides of this value. There was a 10 to 12 fold increase in TAC positive cells within the first 24 hours for PHA and CON-A and an exponential rise in cells expressing the antigen from 24 to 72 hours; however, mitogen stimulated TAC antigen expression is transient and by days 10 to 12 in culture 80% to 90% of receptor expression had disappeared, although cell viabilities remained greater than 95% [26].

Background TAC expression remained low up to 96 hours in culture and rose with time until by day 10 it was expressed on approximately 90% of the cells. This is what is happening when cells are "educated" by FCS to express the IL-2 receptor in the "educated" cell assay [17] by causing G0 to G1 progression probably due to immunogenic and biologically active molecules in the FCS. By day 10 the percentage of cells expressing the TAC antigen in the absence of mitogen exceeded

FIG.4.95 EFFECT OF DIFFERENT DOSES OF CON-A ON SUBSEQUENT EXPRESSION
OF CD-25 AFTER 72 HOURS IN CULTURE

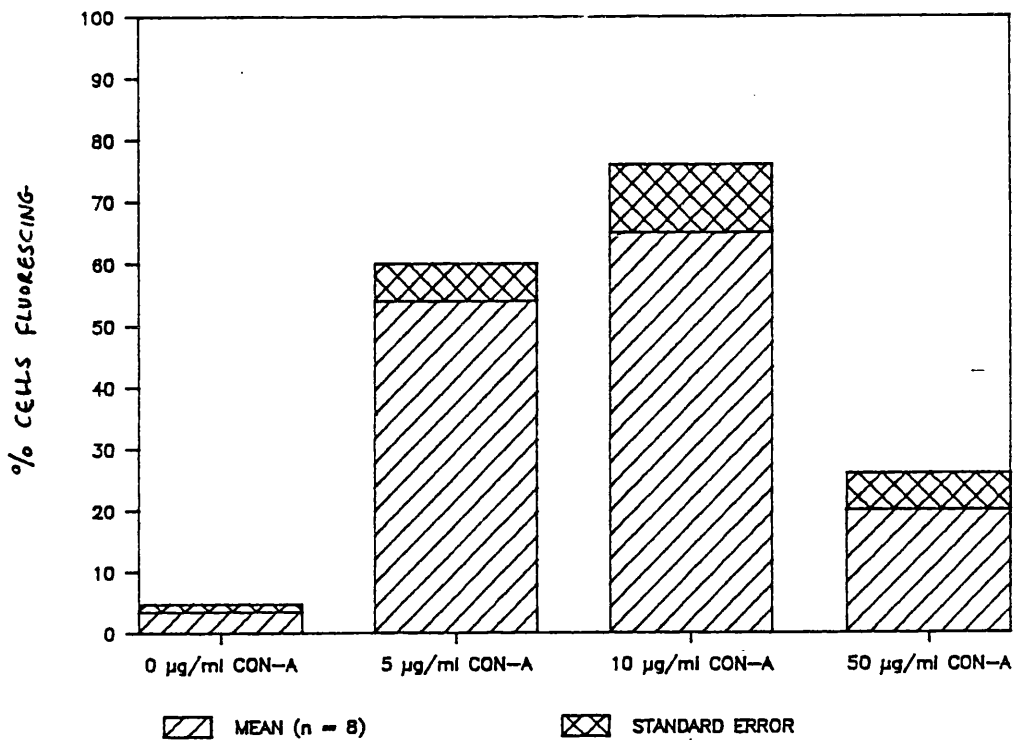
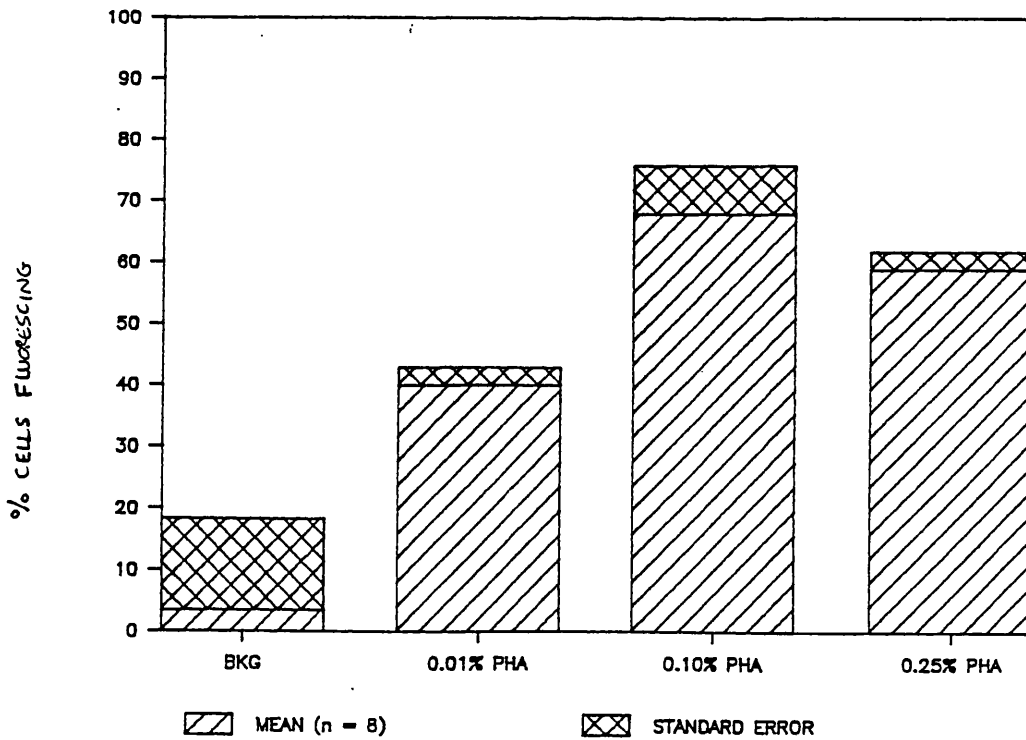


FIG.4.96 EFFECT OF DIFFERENT DOSES OF PHA ON SUBSEQUENT EXPRESSION
OF CD-25 AFTER 72 HOURS IN CULTURE



Showing 10 µg/ml Con-A and 0.1% PHA V:V to be optimal for maximal
CD-25 expression.

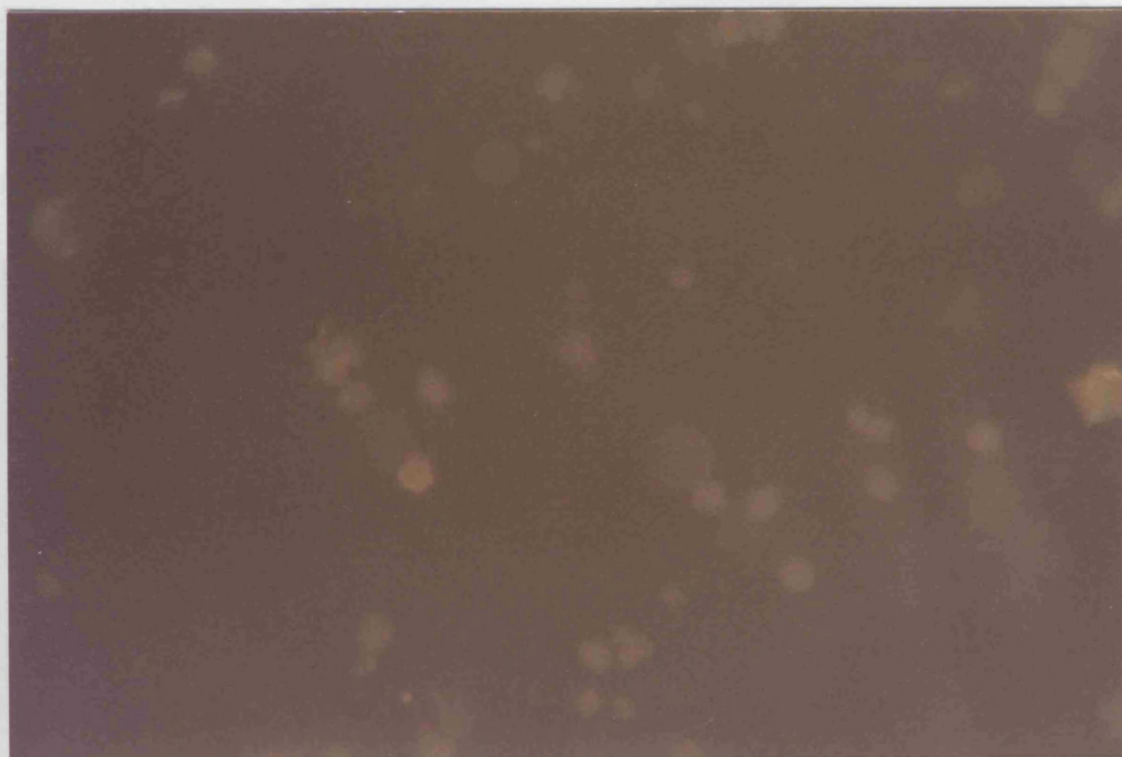


Fig.4.97 : BACKGROUND FLUORESCENCE IN ABSENCE OF ANTI-TAC Mab.
MAGNIFICATION X 400.

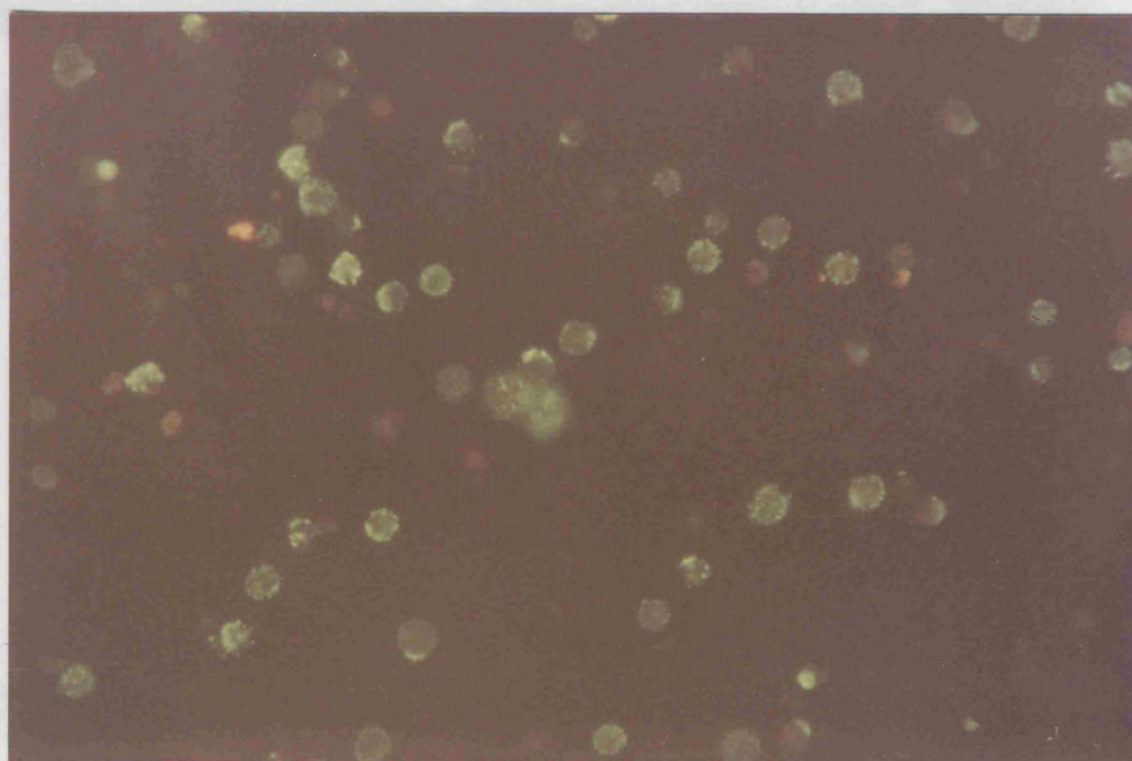


Fig. 4.98: FLUORESCENCE IN PRESENCE OF ANTI-TAC. MAGNIF. X 400.

the TAC expression seen at 72 hours after mitogen stimulation, because whereas the latter culture contains macrocytes and monocytes and T and B cells, only the T and B cells express TAC antigen after mitogen stimulation. But when cells are "selected" to express the TAC antigen

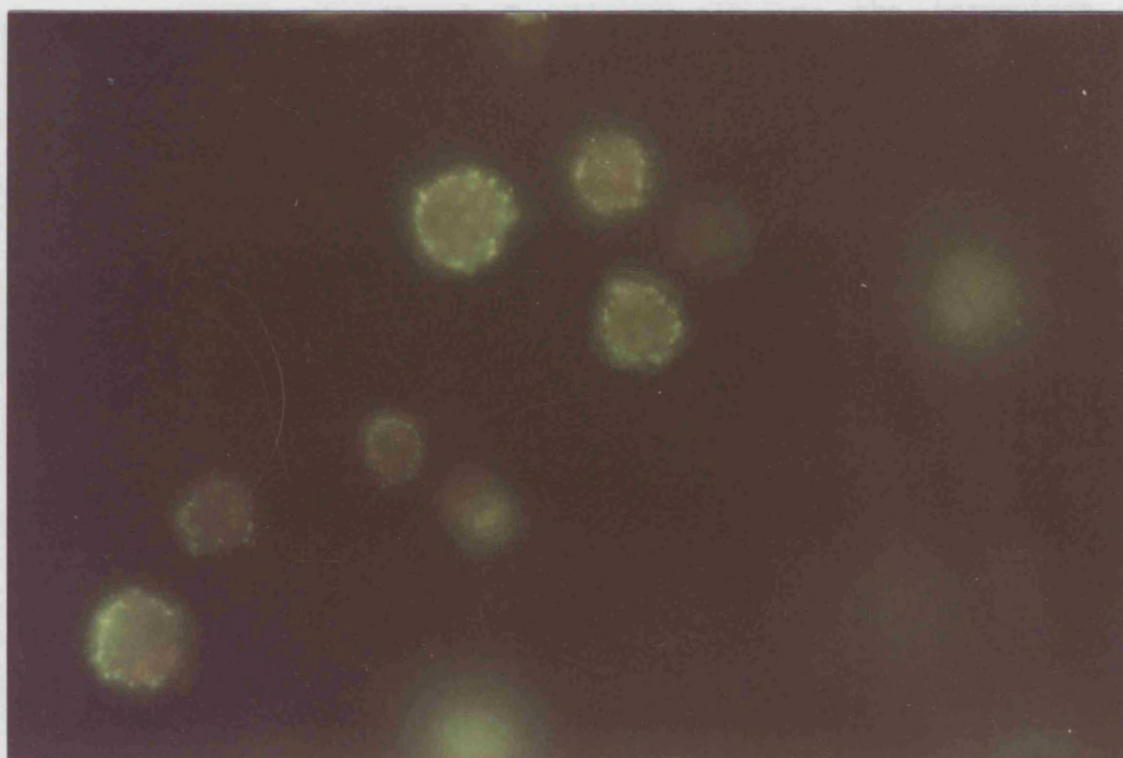


Fig.4.99 : TAC POSITIVE CELLS UNDER HIGH POWER + OIL.

MAGNIFICATION X 1000.

most to 1:1000, for all antigens investigated, with no significant shift of the +FMSA curve to the left, which would have been indicative of an inhibition of antigen expression by FMSA. Thus, antigen expression is not thiol dependent, so this cannot explain the inhibition of proliferation seen.

Also FMSA was found neither to inhibit TAC induction (Fig. 4.110) nor its expression (Fig. 4.111), depending on whether cells

the TAC expression seen at 72 hours after mitogen stimulation, because whereas the latter culture contains monocytes and macrophages and T and B cells, only the T and B cells express TAC antigen after mitogen stimulation, but when cells are "educated" to express the TAC antigen by FCS alone, by day 10 only T cells remain, so the percentage of cells expressing TAC antigen rises (see table 4.31). Of course if a FACS machine was used to compare the density of TAC expression on cells, it would probably be found that TAC was expressed more densely on mitogen stimulated cells than "educated" cells.

Initially the effect of PHMPSA on TAC expression was investigated using one dilution of the Mab (usually neat), however, since it could be argued that subtle differences between +/- PHMPSA treatment could be missed in this way, all further experiments were carried out such that TAC antigen expression was measured using various dilutions of the anti-TAC Mab. All cells had to be greater than 95% viable for this measurement.

Figs. 4.100-4.109 show clearly that the two lines of +/- PHMPSA treatment paralleled each other over all dilutions of Mabs used, i.e. neat to 1:1000, for all antigens investigated, with no significant shift of the +PHMPSA curve to the left, which would have been indicative of an inhibition of antigen expression by PHMPSA. Thus, antigen expression is not thiol dependent, so this cannot explain the inhibition of proliferation seen.

Also PHMPSA was found neither to inhibit TAC induction (Fig. 4.110) nor its expression (Fig. 4.111), depending on whether cells

TABLE 4.31

EDUCATED CELL PHENOTYPE EXPRESSED AS PERCENTAGE OF CELLS STAINING
WITH A PARTICULAR MONOCLONAL ANTIBODY (Mab) IN TWO NORMAL SUBJECTS.

Mab	DAY 0	DAY 10	AFTER 4 DAYS IN CULTURE WITH r-IL-2 (10 IU/ml)	AFTER 4 DAYS IN CULTURE WITH PHA (0.1% V/V)
T3	80%	93%	93%	99%
T4	48%	60%	-	-
T8	31%	40%	-	-
M2	20%	0.5%	0.3%	0%
WR16	60%	62%	-	-
WR17	24%	8%	1%	0%
WR18	20%	94%	-	-
WR19	33%	46%	-	-
TAC	5%	95%	-	-

FIG. 4.100 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD3 EXPRESSION

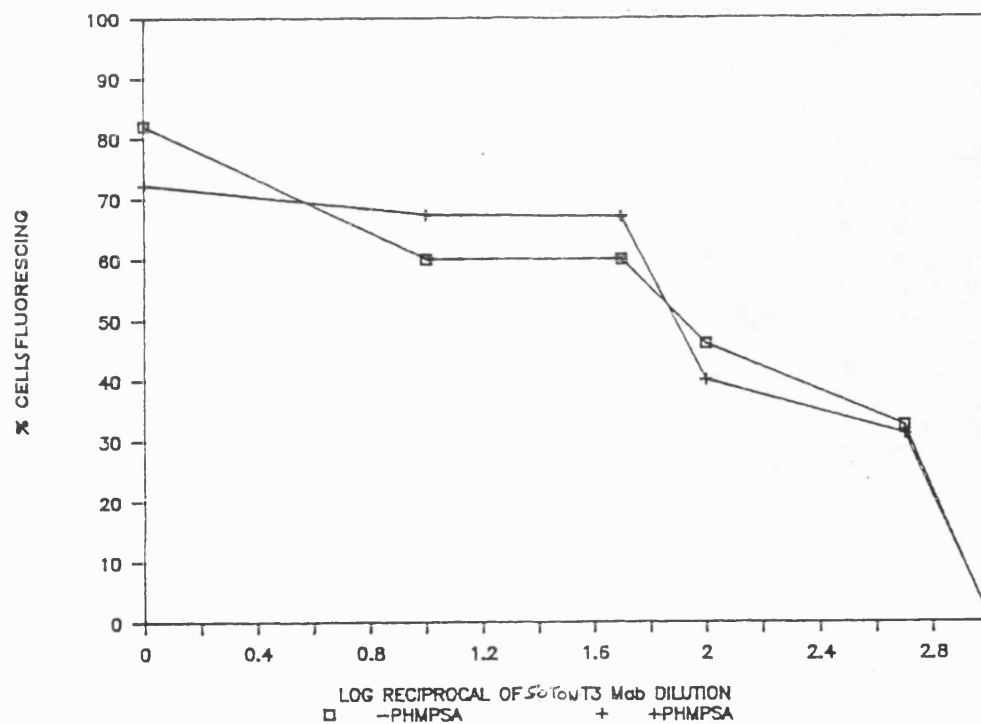
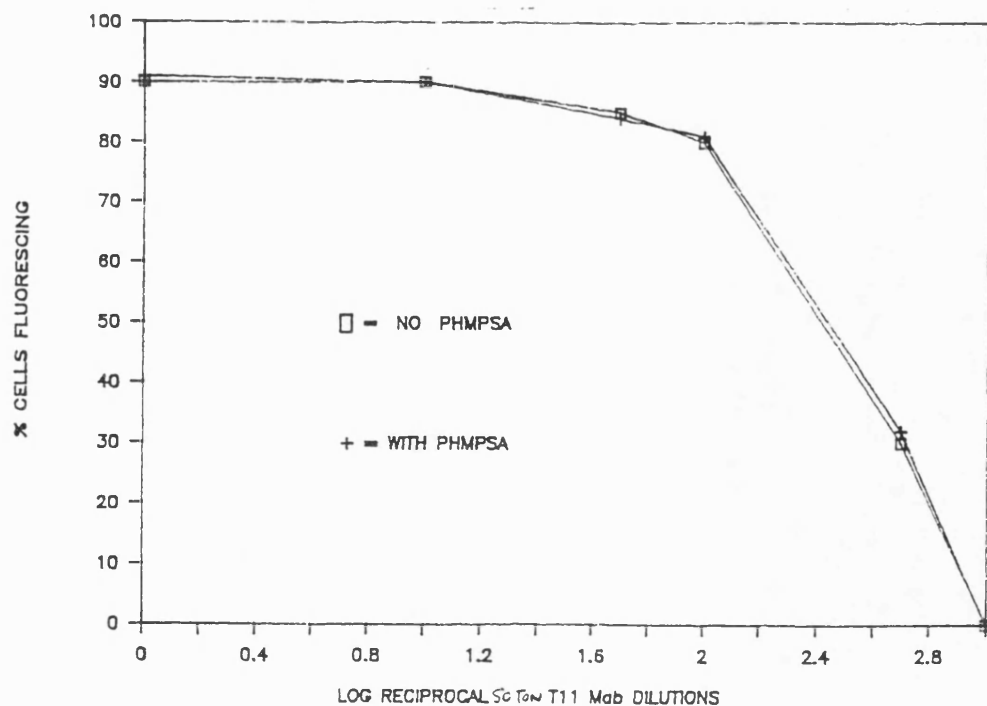


FIG. 4.101 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD2 EXPRESSION



Both graphs show mean of n = 3 individuals. SEM omitted for clarity.

FIG.4.102 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD4 EXPRESSION

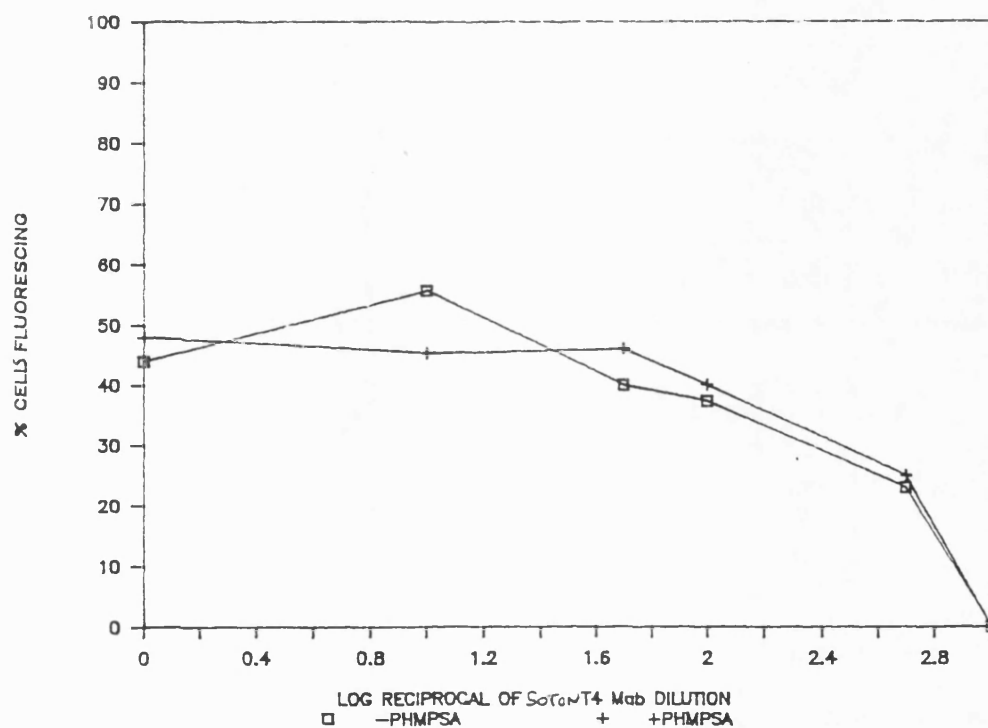
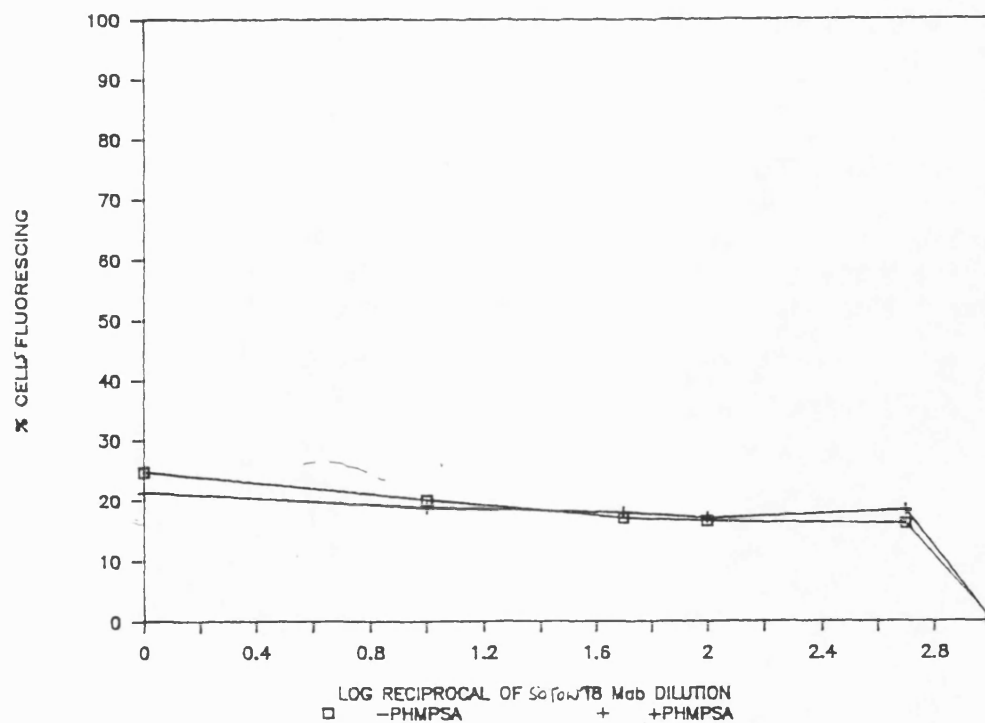


FIG.4.103 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD8 EXPRESSION



Both graphs show mean of n = 3 individuals. SEM omitted for clarity.

FIG.4.104 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD45R EXPRESSION

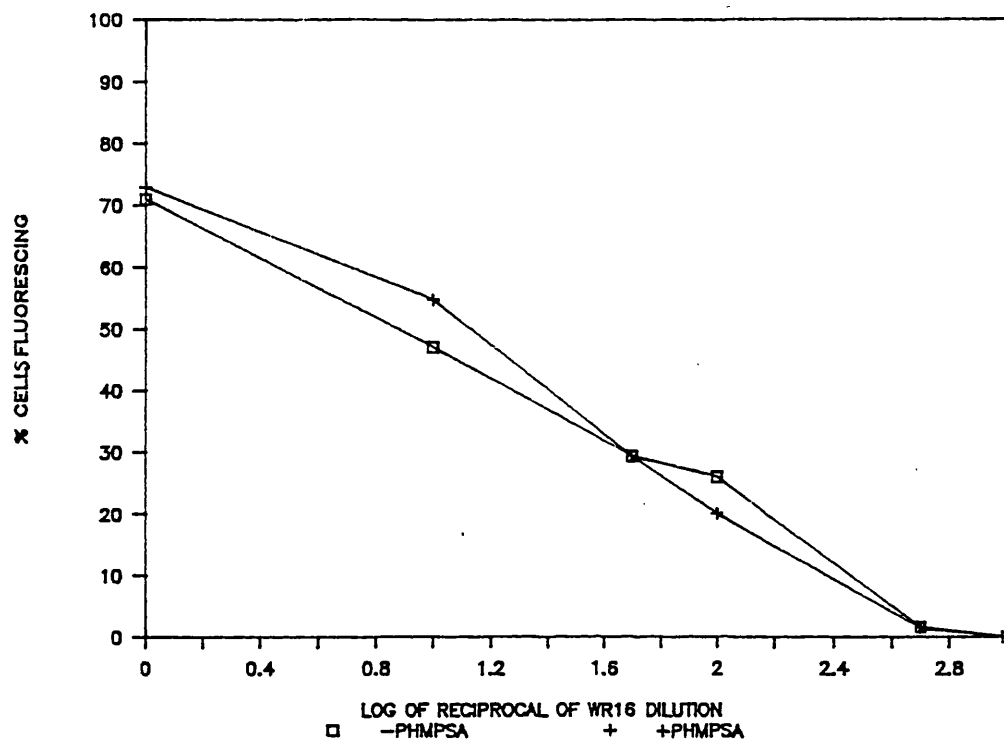
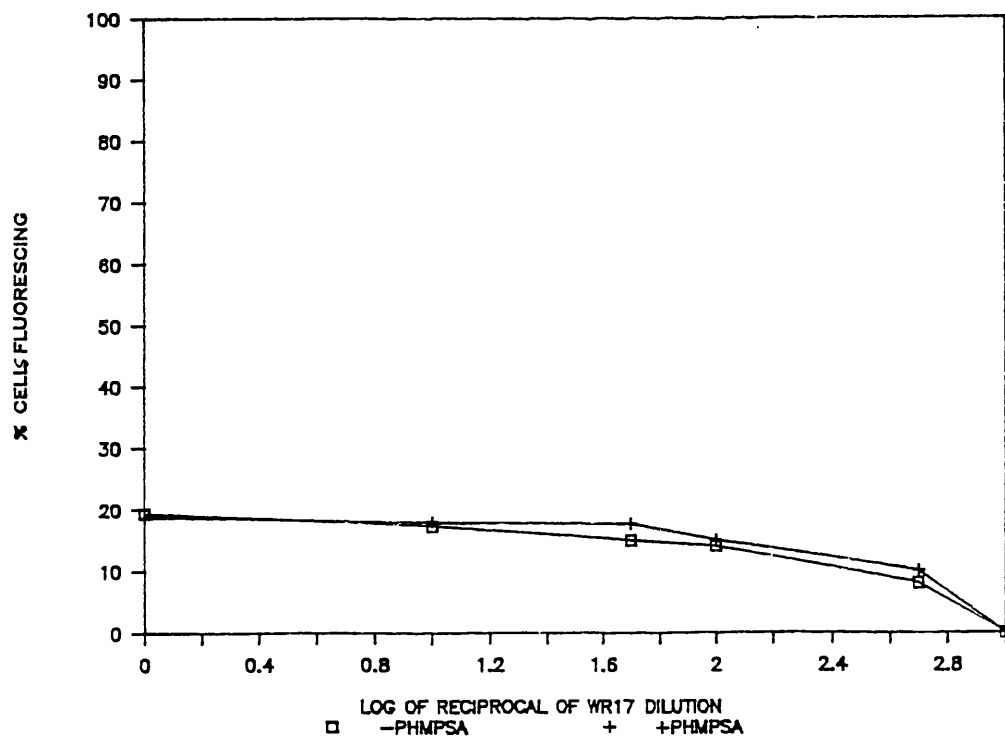


FIG.4.105 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD37 EXPRESSION



Both graphs show mean of n = 3 individuals. SEM omitted for clarity.

FIG. 4.106 EFFECT OF PHMPA TREATMENT (50 μ M) ON EXPRESSION
OF ANTIGEN DETECTED BY WR18

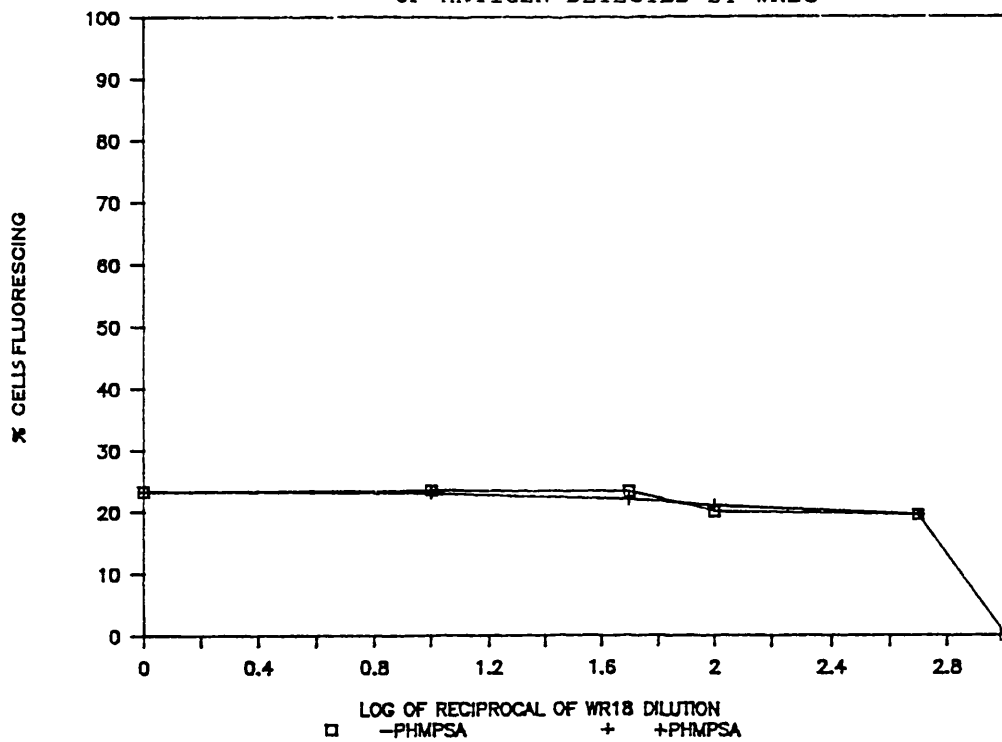
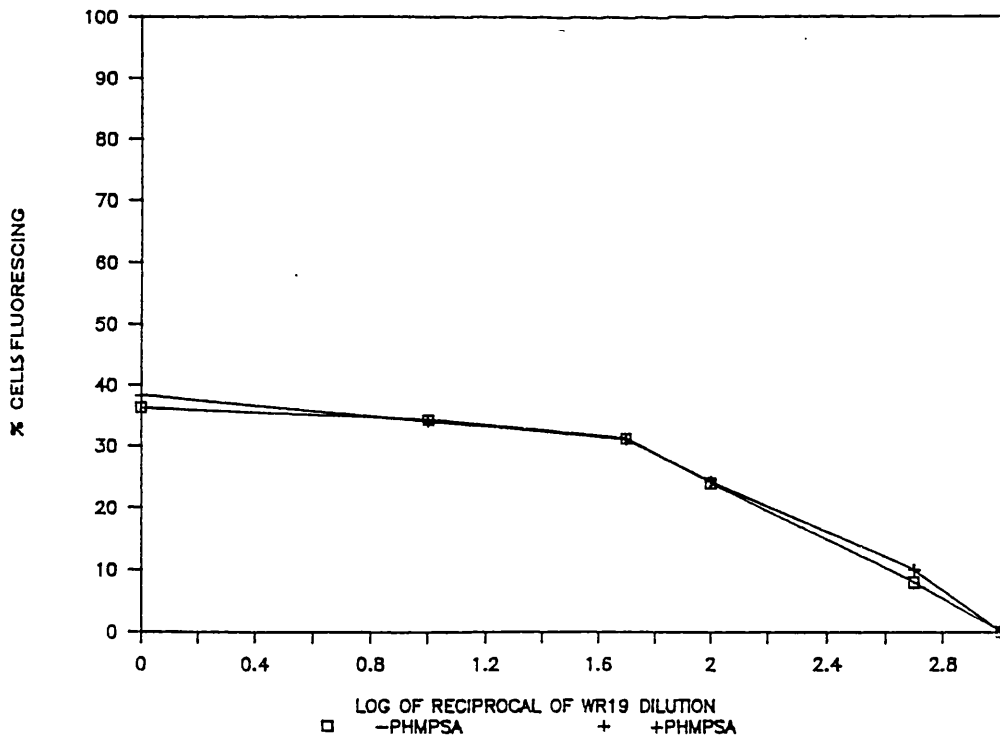


FIG. 4.107 EFFECT OF PHMPA TREATMENT (50 μ M) ON CDW29 EXPRESSION



Both graphs show mean of n = 3 individuals. SEM omitted for clarity.

FIG. 4.108 EFFECT OF PHMPA TREATMENT (50 μ M) ON EXPRESSION OF ANTIGEN
CD 57 MAINLY ON NK CELLS, DETECTED BY HNK-1

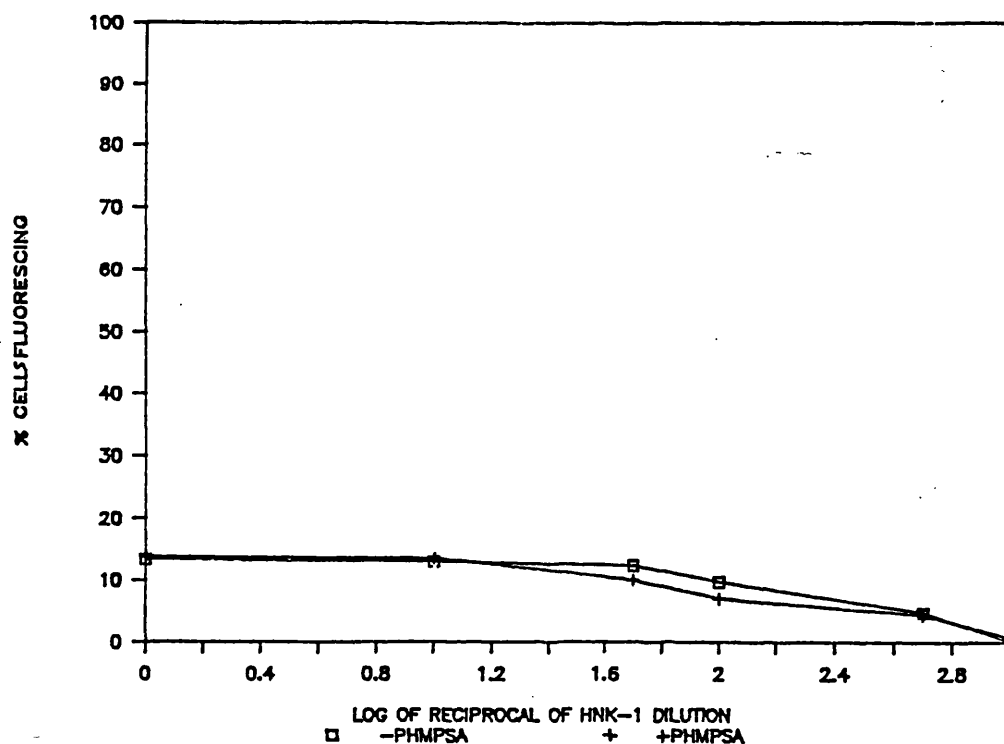
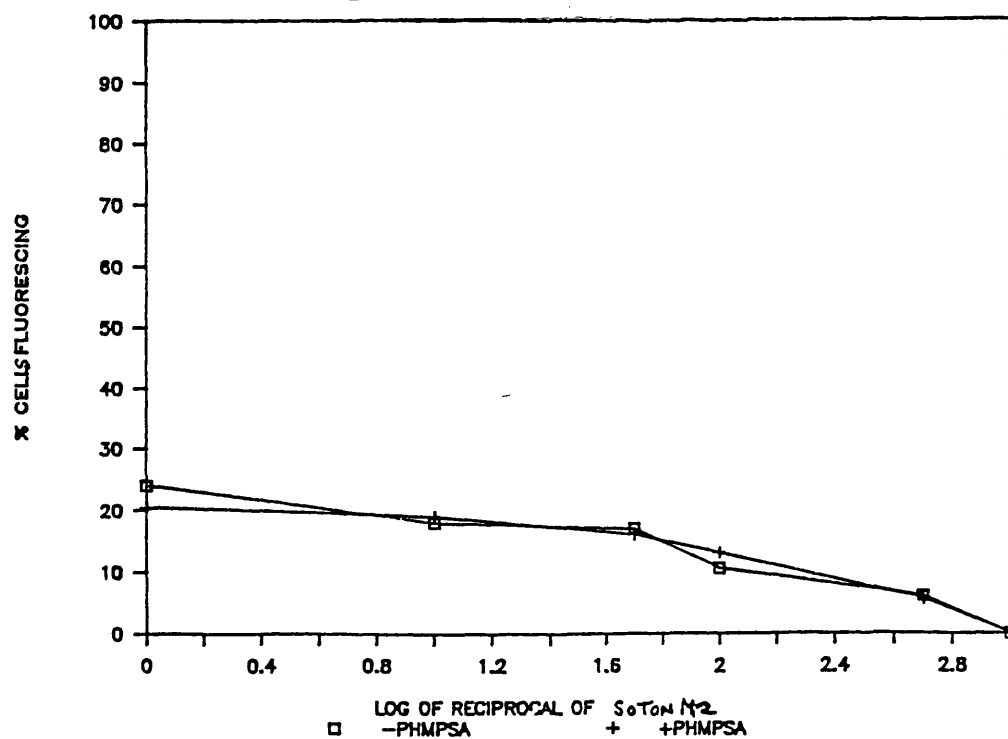


FIG. 4.109 EFFECT OF PHMPA TREATMENT (50 μ M) ON EXPRESSION OF CD11
MAINLY ON MONOCYTES AND MACROPHAGES



Both graphs show mean of n = 3 individuals. SEM omitted for clarity.

FIG 4.110 EFFECT OF PRETREATMENT WITH 50 μ M PHMPSA ON SUBSEQUENT CD25

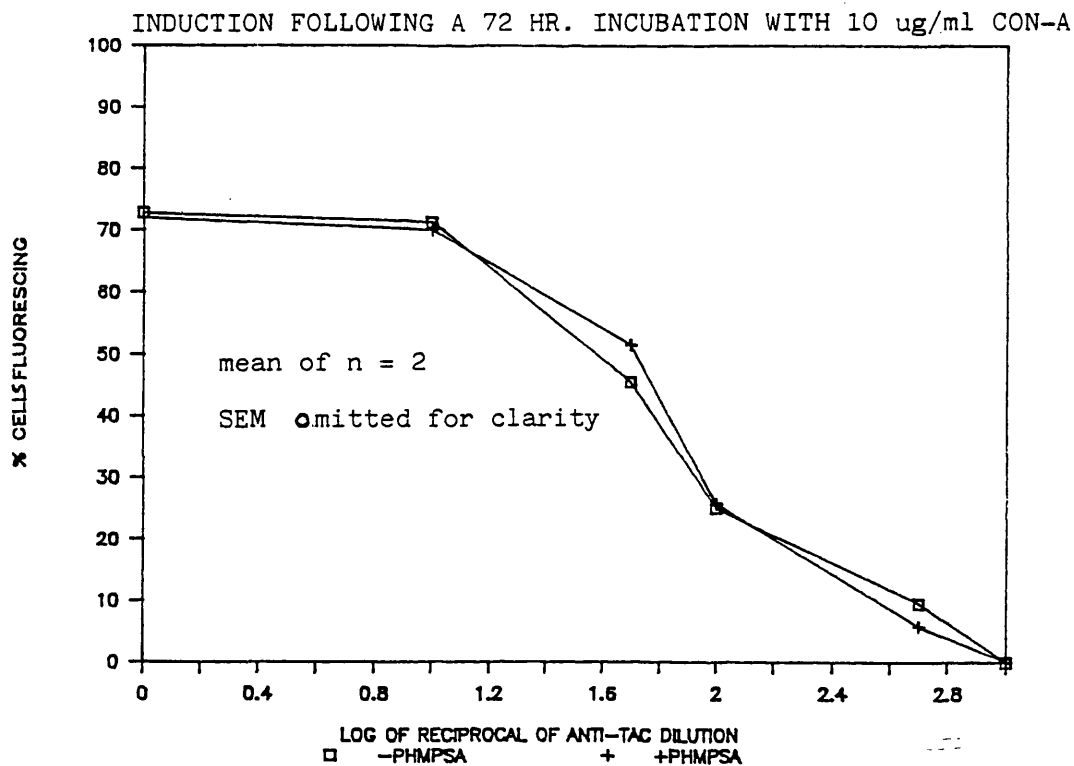
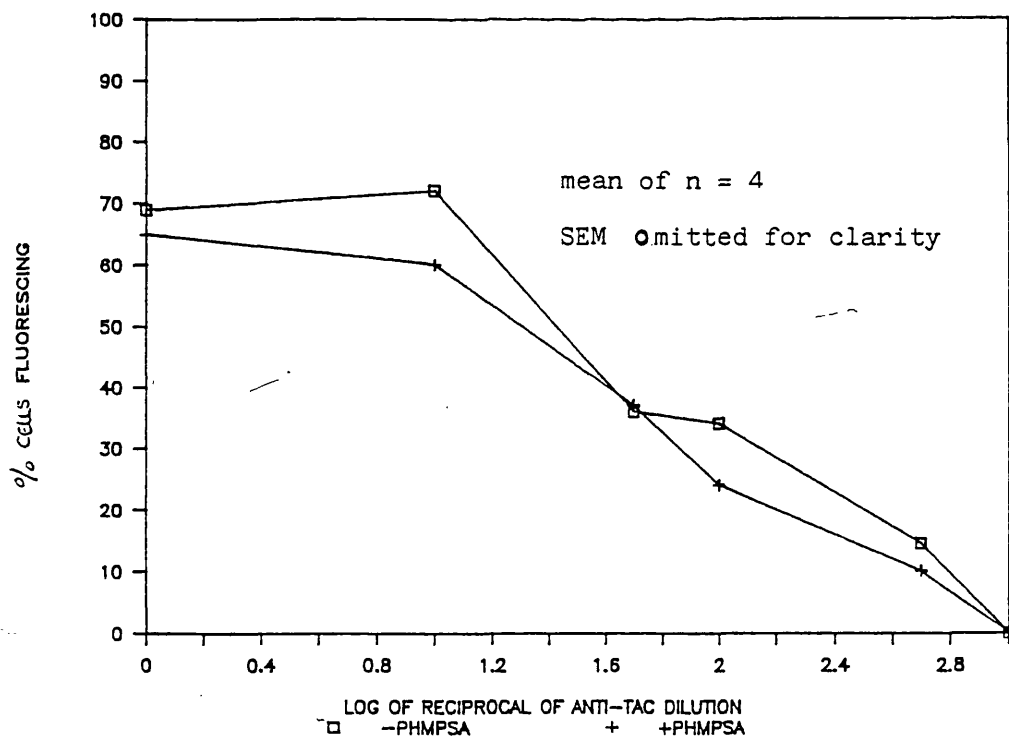


FIG.4.111 EFFECT OF PHMPSA TREATMENT (50 μ M) ON CD25 EXPRESSION AFTER ITS INDUCTION FOLLOWING A 72 HR. INCUBATION WITH 10 μ g/ml CON-A



were treated with PHMPSA prior to mitogen stimulation for 1 hour at 37 °C (induction experiment) or after mitogen stimulation (activity experiment). In both these experiments 10 µg/ml CON-A was used.

In a larger study using neat anti-TAC, the same lack of effect of PHMPSA treatment on IL-2 receptor induction or expression were observed using CON-A and PHA stimulated cells using optimum doses of these mitogens (Figs. 4.112-4.115). Similar results were obtained with suboptimal and superoptimal concentrations of these mitogens (data not shown). Even 100 µM PHMPSA did not abolish TAC induction when cells were stimulated with 0.1% v/v PHA (Fig. 4.116).

Hence, neither an effect on immunoregulatory antigen expression nor on the induction or expression of the TAC antigen can explain the inhibitory effect of PHMPSA on proliferation of PBMNC.

Parallel studies were carried out to see whether the physiological thiol oxidizing agent H_2O_2 had any effect on TAC induction or expression (Figs. 4.117-4.118) using neat anti-TAC Mab. Again no effect was observed even at 10 µM and the reduction in fluorescence at 1 mM H_2O_2 must be disregarded since a 100% cell death occurs here. Similar results were found using dilutions of the anti-TAC Mab up to 1:1000 (data not shown).

Since other workers suggest that IL-2 is important in suppressor cell induction and activity, a failure to express the IL-2 receptor with respect to TAC antigen induction or expression due to SH blockade or oxidation probably cannot explain the defective CON-A induced suppression or short lived suppressor activity seen in RA NSAID.

FIG. 4.112 EFFECT OF 50 μ M PHMPA ON SUBSEQUENT CD25 INDUCTION

FOLLOWING A 72 HR. CULTURE WITH 10 μ g/ml CON-A

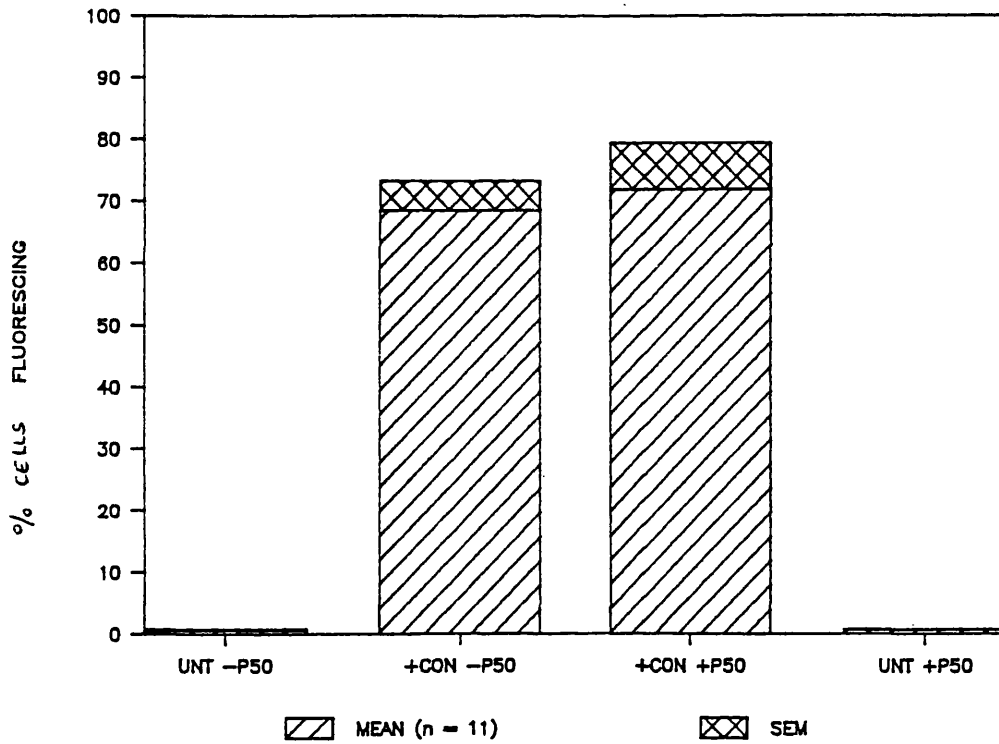


FIG. 4.113 EFFECT OF 50 μ M PHMPA ON SUBSEQUENT CD25 INDUCTION

FOLLOWING A 72 HR CULTURE WITH 0.1% V:V PHA

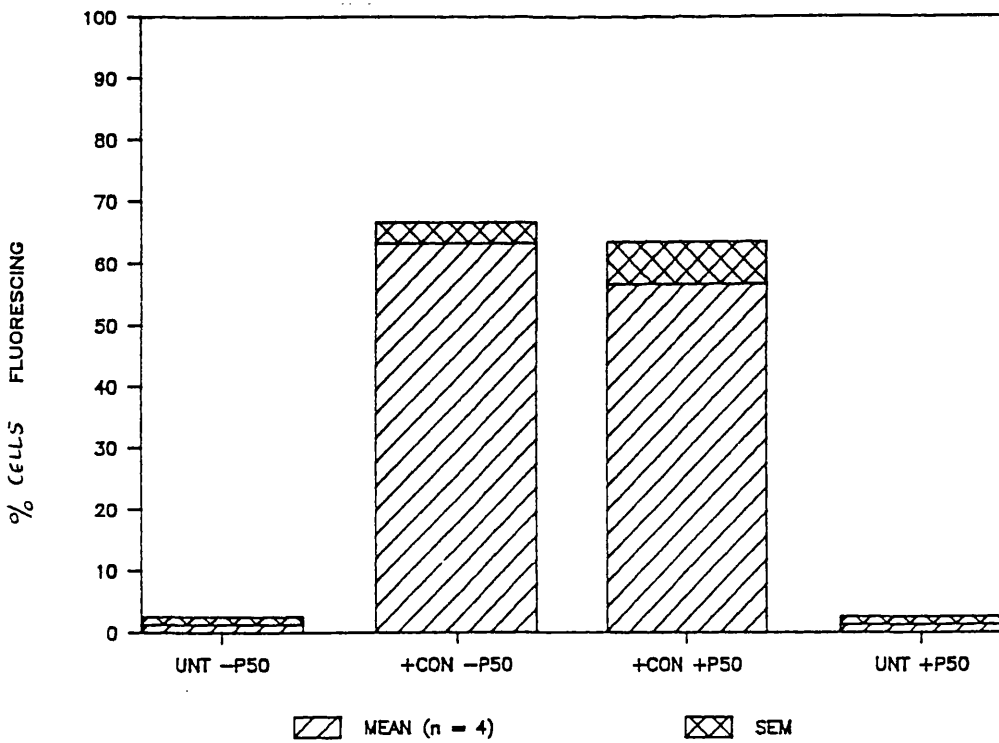


FIG 4.114 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD25 EXPRESSION (DETECTED USING NEAT Mab) FOLLOWING A 72 HR. INCUBATION WITH 10 μ g/ml CON-A

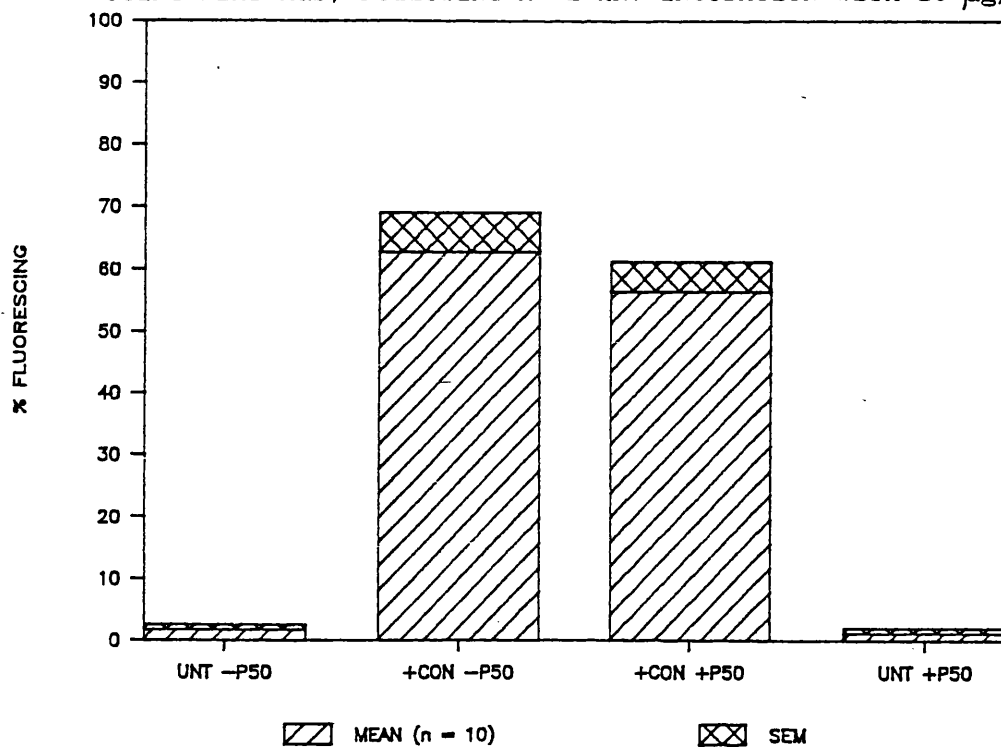


FIG. 4.115 EFFECT OF PHMPA TREATMENT (50 μ M) ON CD25 EXPRESSION (DETECTED USING NEAT Mab) FOLLOWING A 72 HR. INCUBATION WITH 0.1% V:V PHA

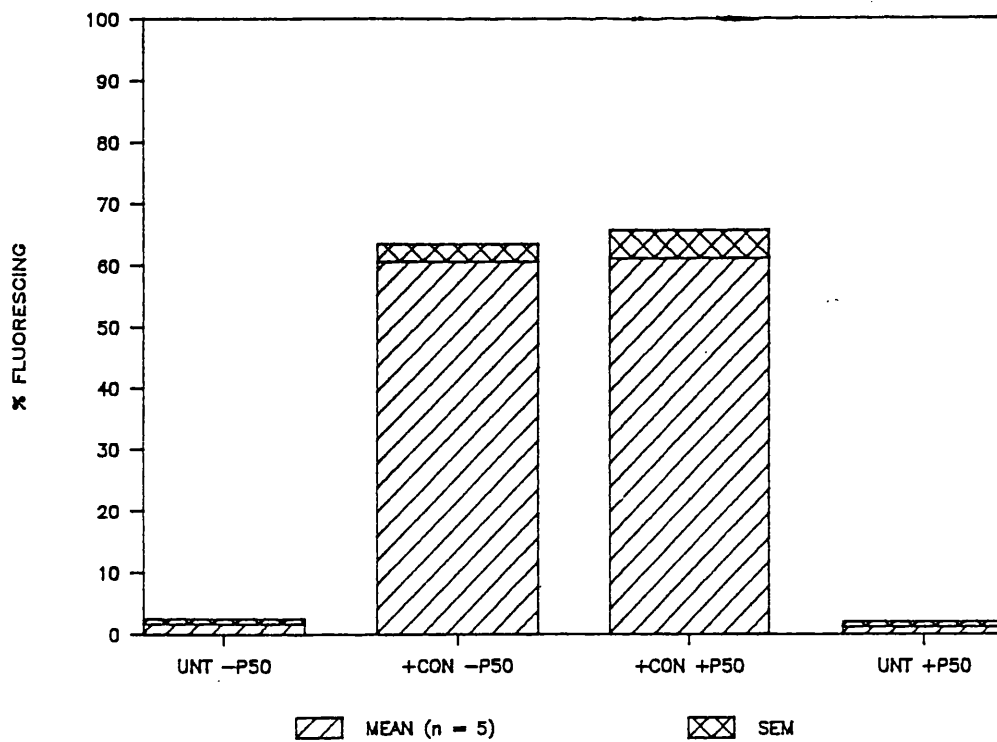


FIG 4.116 EFFECT OF 100 μ M PHMPA ON SUBSEQUENT CD25 INDUCTION

FOLLOWING A 72 HR. CULTURE WITH 0.1% V:V PHA

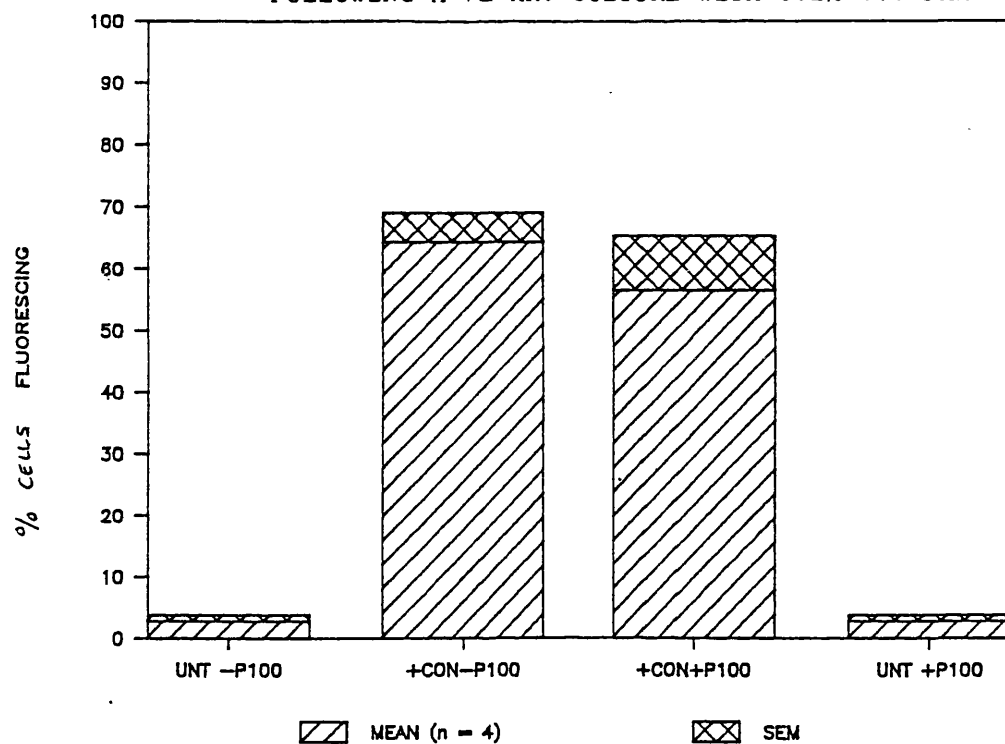


FIG. 4.117 EFFECT OF LEAVING H_2O_2 IN A 72 HR CULTURE
WITH 10 $\mu g/ml$ CON-A ON SUBSEQUENT CD25 INDUCTION

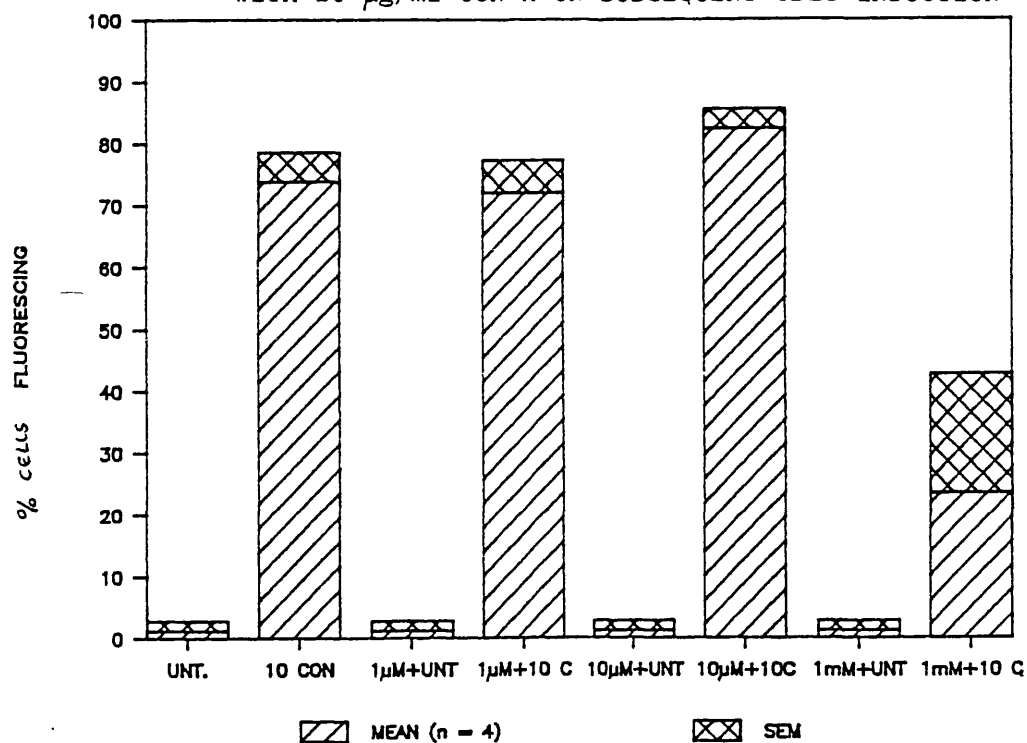
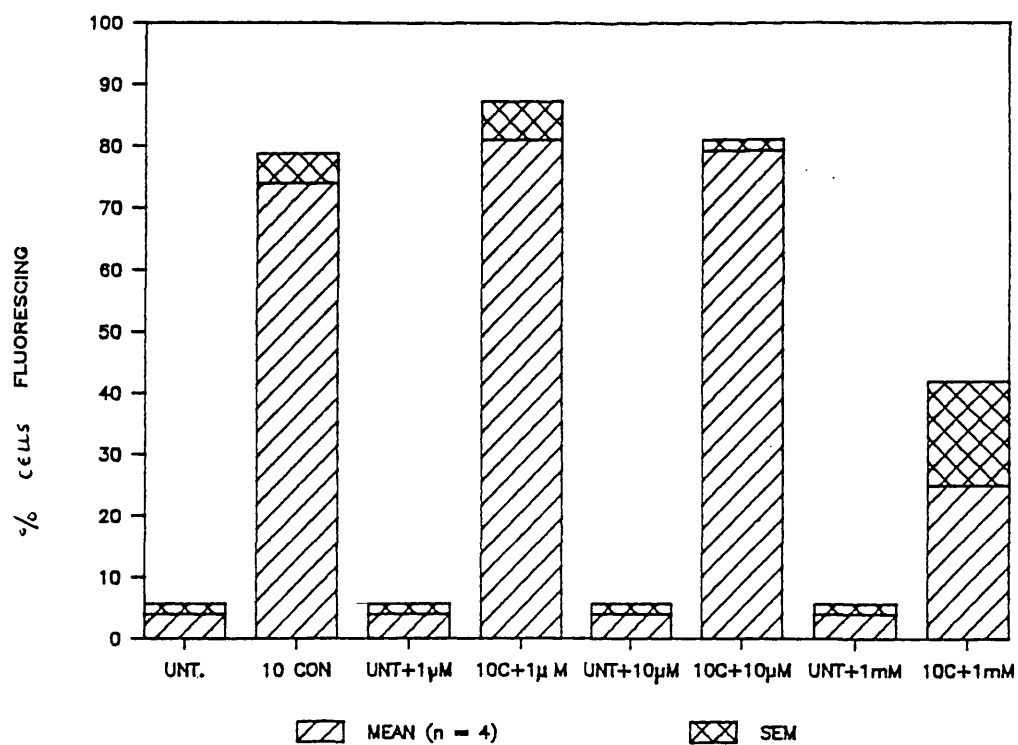


FIG. 4.118 EFFECT OF H_2O_2 ON EXPRESSION OF CD25
AFTER A 72 HR. CULTURE WITH 10 $\mu g/ml$ CON-A



However, this of course tells us nothing about the effect of PHMPSA on the expression of the functionally more relevant P75 subunit of the IL-2 receptor, nor its ability to transduce signals, both of which could conceivably be thiol dependent. The P75 subunit is the more important component of the high affinity IL-2 receptor since it is the protein that transmits the signal into the cell in cooperation with the TAC antigen when associated with it in the high affinity receptor and it can function alone as the intermediate affinity receptor. The intermediate affinity receptor is already expressed on cells prior to mitogen stimulation.

4.23. EFFECT OF PHMPSA ON ABILITY OF "EDUCATED" CELLS TO PROLIFERATE WITH rIL-2.

Since it is also possible to explain the inhibitory effect of PHMPSA on mitogen stimulated proliferation as due to inhibition of high affinity IL-2 receptor formation, signal transduction from the IL-2 receptor into the cells, or inhibition of IL-2 binding to its receptor, systems for investigating these possibilities were devised.

"Educated" cells, expressing the high affinity IL-2 receptor, which can subsequently proliferate when rIL-2 or IL-2 containing supernatants are added, causing progression through the cell cycle from $G1 \rightarrow S \rightarrow G2 \rightarrow M$ [17,17a], offers a system for investigating these possibilities.

50 μM and 100 μM PHMPSA were left for 10 days in a culture medium with the cells being "educated" to see their effect on induction of

"educated" cells as assessed by their subsequent ability to proliferate in response to rIL-2. Table 4.32 shows results expressed as mean relative percent proliferation relative to cultures without PHMPSA treatment. Inhibition was observed throughout the range of rIL-2 concentrations known to stimulate the high and intermediate receptors, but it was thought that this methodology could be criticised since it could be argued that the inhibition seen was due to an effect of PHMPSA on components in the medium and not a direct effect on signal transduction or IL-2 binding, or high affinity receptor formation.

In testing the effect of PHMPSA on the activity of "educated" cells which already express the high affinity IL-2 receptor, increased suppression of proliferation of the "educated" cells was observed when 50 μ M PHMPSA was left in a 4 days culture with "educated" cells and a range of IL-2 that would stimulate high and intermediate affinity IL-2 receptor (Table 4.33). However, this increased inhibition could again be argued to be the result of PHMPSA inhibiting components in the medium together with possibly inhibiting IL-2 binding to its receptor or signal transduction.

To overcome these criticisms, instead of leaving PHMPSA in the 10 days culture, PBMNC were treated with PHMPSA and then it was washed off, prior to the cells being "educated", to test for an effect on "induction" of "educated" cells. Instead of leaving PHMPSA in a 4 days culture after "educated" cells were made, "educated" cells were post-treated with PHMPSA which was then washed off, prior to the cells being set up to proliferate in response to a range of rIL-2 known to

TABLE 4.32

EFFECT OF LEAVING PHMPA IN WITH CELLS BEING "EDUCATED" THROUGHOUT THEIR 10 DAY CULTURE PERIOD ON THEIR SUBSEQUENT ABILITY TO PROLIFERATE IN RESPONSE TO r-IL-2. RESULTS ARE EXPRESSED AS MEAN RELATIVE % PROLIFERATION RELATIVE TO CULTURE WITHOUT PHMPA IN 6 NORMAL SUBJECTS.

[r-IL-2] (IU/ml)	50 μ M PHMPA	100 μ M PHMPA
1000	- 98%	- 100%
500	- 78%	- 100%
100	-	- 100%
50	- 70%	-
10	- 74%	- 100%
3	- 73%	- 100%
1	- 54%	-
0.3	- 66%	- 100%
0.1	- 74%	-
0.03	- 77%	-
0.01	- 68%	-
0.003	- 99%	-
0.001	- 70%	-

TABLE 4.33

EFFECT OF LEAVING 50 μ M PHMPA IN WITH 4 DAY CULTURES OF "EDUCATED" CELLS + r-IL-2. RESULTS ARE EXPRESSED AS MEAN RELATIVE % PROLIFERATION RELATIVE TO CULTURE IN THE ABSENCE OF PHMPA IN THREE NORMAL SUBJECTS.

[r-IL-2] (IU/ml)	RELATIVE % PROLIFERATION
1000	- 97%
500	- 99%
100	- 85%
50	- 95%
10	- 83%
1	-100%
0.1	-100%
0.01	-100%

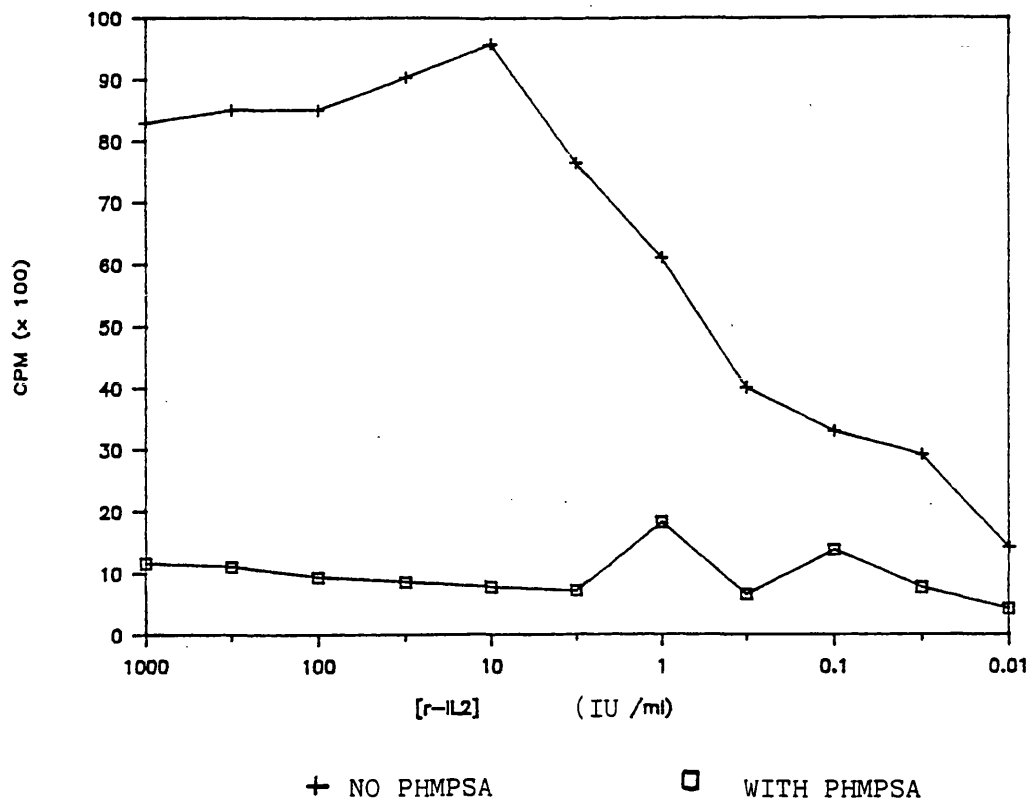
be able to stimulate high and intermediate IL-2 receptors in order to test the effect of PHMPA on the expression of "educated" cell "activity".

The resultant data can be shown in three ways, which are illustrated in relation to results obtained from the various experiments:

The first way is to show average CPM for all data \pm PHMPA, for all individuals, however this becomes meaningless due to the enormous individual variation in CPM seen for similar concentrations of rIL-2. The only way to overcome this is to show individual data for individual subjects, but this would be laborious, so one individual has been shown to give an example of the data obtained after post-treatment of "educated" cells with 50 μ M PHMPA (Fig. 4.119), showing suppression of proliferation over the all range of rIL-2 concentrations from 0.01 to 1000 IU/ml.

A second approach is to express the data as percentage of maximum counts in the non-PHMPA treated cells per individual and then graphically represent the average of these values for all individual studies as two lines in one graph, as shown in fig. 4.120, depicting the effect of post-treatment of "educated" cells on subsequent ability to proliferate to a range of rIL-2 concentrations. This method of showing the data clearly shows suppression throughout the range 0.01 to 1000 IU/ml rIL-2, which cannot be an artefactual effect of PHMPA on medium components since it was thoroughly washed off. However, showing the data in this way tends to indicate that there was more

FIG. 4.119 EFFECT OF POST-TREATMENT WITH 50 μ M PHMPSA ON SUBSEQUENT
PROLIFERATION IN RESPONSE TO rIL-2 IN ONE NORMAL FEMALE



Data show actual CPMs.

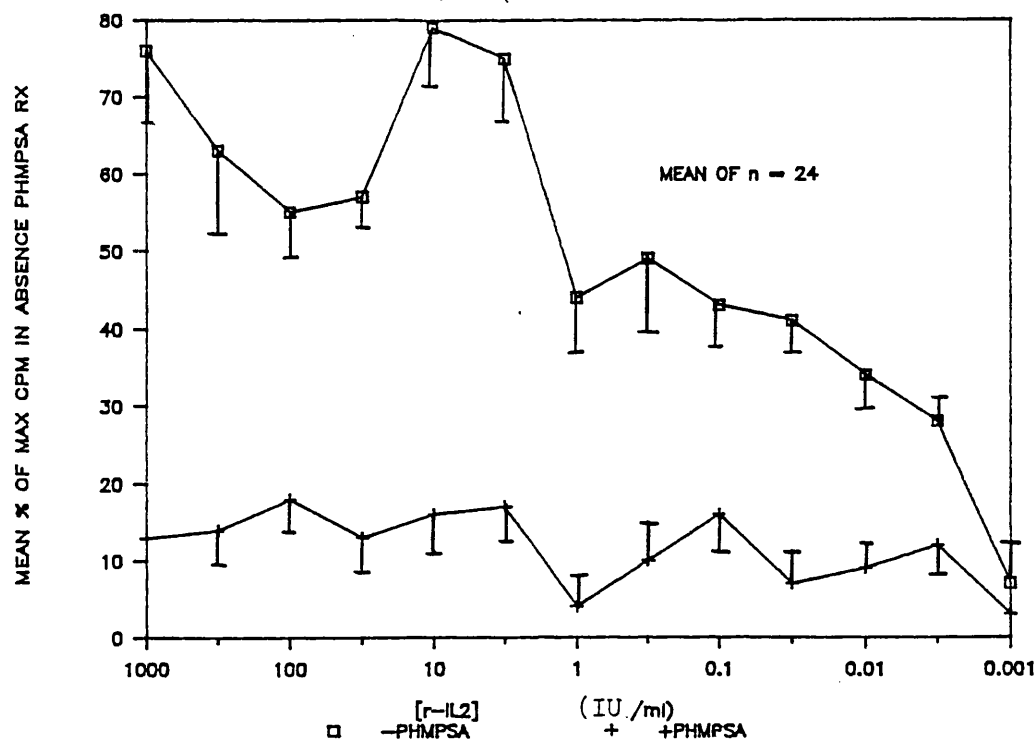
At 1000 IU/ml rIL-2 relative % proliferation was -86% after PHMPSA treatment

At 1 IU/ml rIL-2 relative % proliferation was -70% after PHMPSA treatment

At 0.01 IU/ml rIL-2 relative % proliferation was -70% after PHMPSA treatment.

FIG. 4.120 EFFECT OF POST-TREATMENT OF EDUCATED CELLS WITH μ M PHMPSA

-THEN WASHED OFF- ON SUBSEQUENT PROLIFERATION IN RESPONSE TO rIL-2



At 1000 IU/ml rIL-2 mean relative % proliferation was -83% after PHMPSA Rx

At 1 IU/ml rIL-2 mean relative % proliferation was -91% after PHMPSA Rx

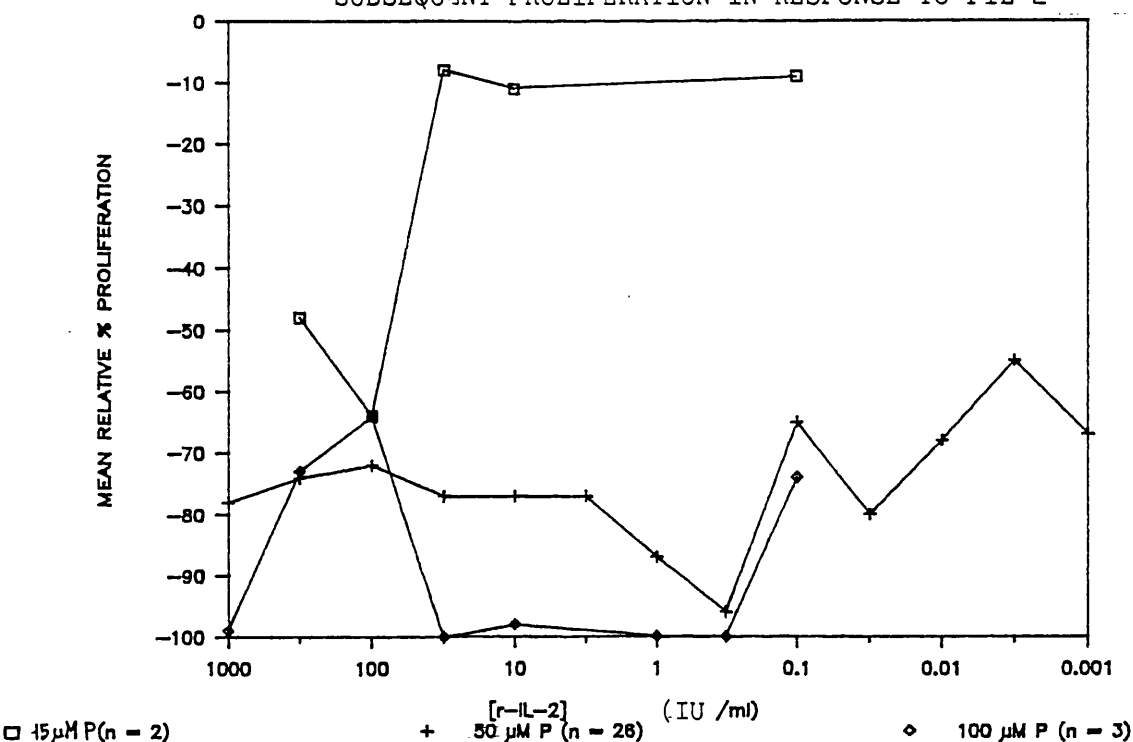
At 0.001 IU/ml rIL-2 mean relative % proliferation was -57% after PHMPSA Rx.

suppression observed using higher doses of rIL-2 than at lower doses, when in fact this was not the case, since the mean relative percent inhibition of proliferation was found to be similar at all concentrations of rIL-2.

What is of more importance is the actual relative percent inhibition of proliferation at individual doses of the recombinant IL-2 in individuals. This third method of depicting the data results in the expression of the data as one line on a graph. This method was used to depict the data obtained when the cells were pretreated with PHMPSA prior to being "educated" (Fig. 4.121). It shows more clearly that there is in fact equal suppression at all doses of rIL-2 used (as was seen for post-treatment too). The data for 50 μ M PHMPSA is more meaningful since it represents the average data from 26 normals as opposed to much fewer data for the other two concentrations of PHMPSA used. What it does show, however, is a dose response effect to PHMPSA, i.e. less suppression at 15 μ M PHMPSA and more at 100 μ M compared to 50 μ M PHMPSA.

The phenotype of the cells being "educated" over a 10 days period shown in table 4.31 clearly shows the percentage of T cells to increase (both CD_4^+ and CD_8^+ increase as do cells interacting with WR16 and WR19); however the percentage of monocytes and B cells fall dramatically. Dramatic increases in TAC and DR antigen expression are observed indicative of T cell activation by FCS in the medium. Four days after culture with rIL-2 the percentage of monocytes and B cells falls further to the extent that only T cells remain. This fall is seen to a greater extent if "educated" cells are allowed to

FIG. 4.121 EFFECT OF PRETREATMENT WITH PHMPSA (15, 50, 100 μ M)
ON INDUCTION OF EDUCATED CELLS, AS ASSESSED BY THEIR
SUBSEQUENT PROLIFERATION IN RESPONSE TO rIL-2



Increasing concentrations of PHMPSA caused increased suppression of educated cells induction, and this was observed to approximately the same extent following 50 μ M PHMPSA pretreatment for all the rIL-2 concentrations used.

SEM were omitted for clarity.

proliferate in response to the mitogen PHA instead of rIL-2.

Looking at the phenotype of the "educated" cells before and after the pretreatment with 50 μ M PHMPA (Table 4.34), similar results are obtained as seen in the fluorescent study, i.e. PHMPA had no effect on the percentage of cells expressing CD3, CD4, CD8, CD11b, CDW29, CD37, HLA-DR antigen, CD45R and CD25 antigen.

Similar results were obtained if PHMPA was left in the 10 days culture period (data not shown).

Experiments were carried out to see if the physiological oxidizing agent H_2O_2 paralleled those results observed with PHMPA. It was considered more appropriate to leave H_2O_2 in the 10 day culture period whilst cells were being "educated" to express the IL-2 receptor, since this is more akin to the situation in RA NSAID. Again, as in the fluorescent study, no inhibition of TAC induction was observed, only at 10 μ M H_2O_2 and beyond, at which point 50% of the cells are dead and 100% of the cells are dead respectively (Table 4.35).

The results from the "educated" assay could be interpreted in several ways;

1) inhibition of proliferation in experiments to show the effect of PHMPA on the "activity" of "educated" cells after the IL-2 receptor has been formed, could mean that rIL-2 binding to the receptor is inhibited by PHMPA, or PHMPA inhibits signal transduction;

2) inhibition of proliferation in experiments to show the effect

TABLE 4.34

EFFECT OF PRETREATMENT WITH/WITHOUT 50 μ M PHMPA ON RESULTING PHENOTYPE OF EDUCATED CELLS AT DAY 10. RESULTS ARE EXPRESSED AS PERCENTAGE OF CELLS STAINING WITH A PARTICULAR Mab (MEAN FROM TWO NORMAL SUBJECTS).

Mab	NO PHMPA	50 μ M PHMPA
T3	93%	92%
T4	60%	59%
T8	40%	39%
M2	0.5%	0.5%
WR16	62%	61%
WR17	8%	11%
WR18	94%	93%
WR19	46%	43%
TAC	95%	94%

TABLE 4.35

EFFECT OF LEAVING H_2O_2 IN 10 DAY CULTURE ON IL-2R EXPRESSION ON EDUCATED CELLS BY DAY 10.

$\pm H_2O_2$ (μ M)	0	1	10	50
% POSITIVE CELLS STAINING WITH DAKO-IL-2R Mab	95	93	30	0
% CELL VIABILITY AT DAY 10	95	95	40	0

of PHMPA on the "induction" of "educated" cells before the IL-2 receptor has been formed, could mean that PHMPA blocks important thiol groups on the P75 protein necessary for its interaction with the TAC antigen for forming the high affinity IL-2 receptor, if the latter is a disulphide linked heterodimer. This condition would therefore be analogous to that of the disulphide linked heterodimeric structure of CD6-CD8 dimer on immature thymocyte, or the Ti antigen of the T-cell receptor [27] and similar to homodimers such as the CD8 antigen [28] and CD28 antigen [29] which is revealed under reducing conditions (addition of 2-ME) in SDS electrophoresis. Intramolecular disulphide bridges (cysteinyll residues) have been proposed to be important in the membrane organisation, structure, function and activation of many cell surface receptors coupled to their effector system via G proteins (e.g. mammalian β_1 and β_2 adrenergic receptors; hepatic glucagon receptors; opiate receptors; IL-2 (TAC Ag), IL-3, LH/hCG receptors [30]). A disulphide bond, but not SH groups, seems to be important in the function of the C3bi receptor (CD11) for EA rosette formation and immune adherence [31]. If the high affinity IL-2 receptor is not disulphide linked, the former two arguments in 1) could still be applied here. Further experiments were designed to clarify which of these views is correct.

4.24. EFFECT OF PHMPA ON ABILITY OF rIL-2 TO BIND TO THE IL-2 RECEPTOR.

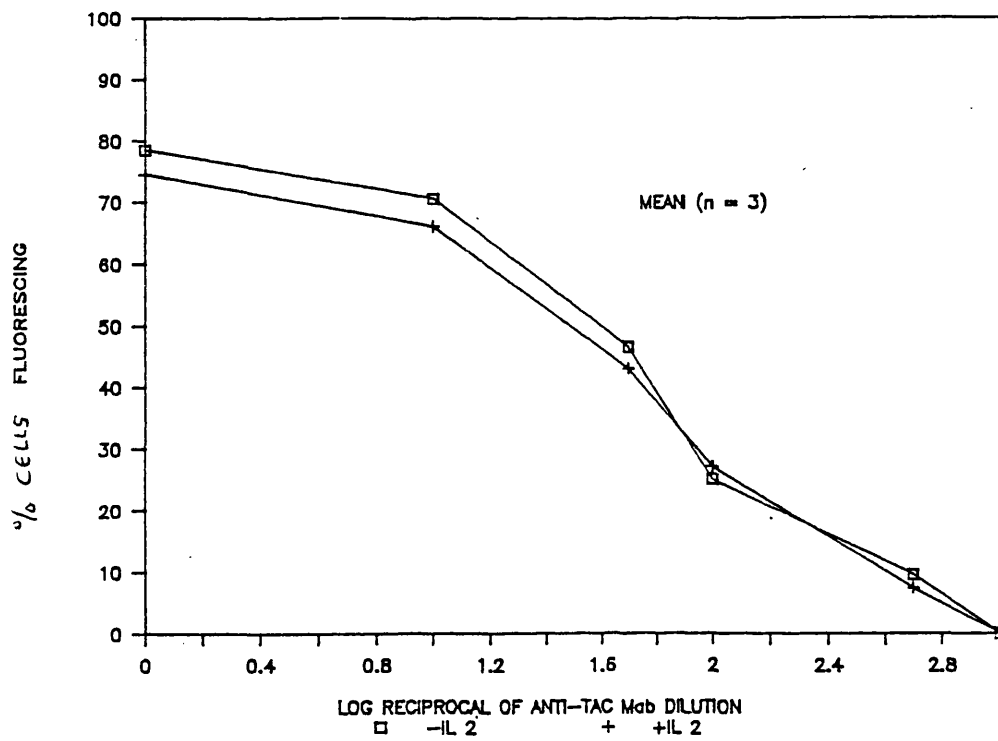
In order to test whether PHMPA inhibits rIL-2 binding to its receptor, PBMNC were treated with 10 μ g/ml CON-A for 72 hours in order

to induce the high affinity rIL-2 receptor, cells were then harvested and treated for 1 hour at 37 °C with/without 50 μ M PHMPSA and then washed and incubated with 1 μ g/ml rIL-2 (a concentration which should be sufficient to bind all IL-2 receptors present). The cells were washed and then treated with a range of dilutions of anti-TAC from neat to 1:1000 dilutions, so that any subtle differences following PHMPSA treatment would not be missed.

However, the experiment had to be abandoned owing to the fact that rIL-2 was found not to block anti-TAC binding (Fig. 4.122), even at doses as high as 10 μ g/ml. It could be that excess anti-CD25 Mab was used with respect to rIL-2, despite the evidence that the low affinity receptor has a forty-fold greater affinity for IL-2 than anti-CD25 [32a], and the high affinity receptor has a thousand-fold greater affinity for IL-2 (k_d 10^{-12} M) [32b] than anti-CD25 (k_d 10^{-9} M) [32c], thus favouring IL-2 binding. (Therefore one would need considerably excess anti-CD25 to prevent competition with rIL-2). However, a more likely explanation is that anti-CD25 binds to a slightly different epitope on the IL-2 receptor than IL-2. This is not surprising in view of the fact that the literature regarding the DAKO anti-TAC states "DAKO anti-IL-2R binds to the IL-2R without inhibiting its function", and in fact the vast majority of commercially available anti-TAC Mabs are said not to interfere with IL-2 binding to its receptor, with the exception of Becton Dickinson's anti-TAC Mab, for which it is stated that "rIL-2 blocks binding of anti-IL-2R to PHA activated T cells". Data for this statement was supplied to this company by Dr. Steven Gillis, who found that a 10 to 50 fold molar excess of IL-2 over

FIG. 4.122

EFFECT OF R-IL-2 ON ANTI-TAC BINDING



SEM were omitted for clarity.

Showing rIL-2 did not inhibit anti-TAC binding.

radiolabelled 2A3 (the antibody), will inhibit binding to 70% at 100 nM IL-2. However, at such high concentrations of rIL-2 one could argue that the effect is not specific.

If time had permitted, experiments would have been repeated using this Mab, which was not available at the time. Likewise, another author found purified IL-2 in high concentration - $> 125 \text{ ng}/10^6$ cells - blocked binding to $> 85\%$, rising to 90% using $500 \text{ ng}/10^6$ cells, of radiolabelled IgG2a subclass anti-TAC (made by the method of Uchiyama [22]) to PHA lymphoblasts; however low concentrations - $< 20 \text{ ng}/10^6$ cells - had very little effect [33]. It has also been stated in the literature that some anti-TAC Mabs that can block proliferation in response to added rIL-2, can still bind to the β chain of the IL-2 R after rIL-2 has been bound, e.g. saturable levels of IL-2 failed to inhibit ART 18 (a Mab said to detect rat TAC Ag) binding, said to be because different binding sites for IL-2 and ART Mab on the same unit structure of the cell are used. The authors of this paper suggest that Mabs that block the function of the IL-2 R - i.e. block IL-2 binding and capacity to proliferate in response to rIL-2 - do not necessarily have to bind to the receptor itself, e.g. they could bind to another T-lymphoblast specific antigenic determinant, and indirectly affect the IL-2 R by - for instance - co-patching and eliminating the receptor from the cell surface [34].

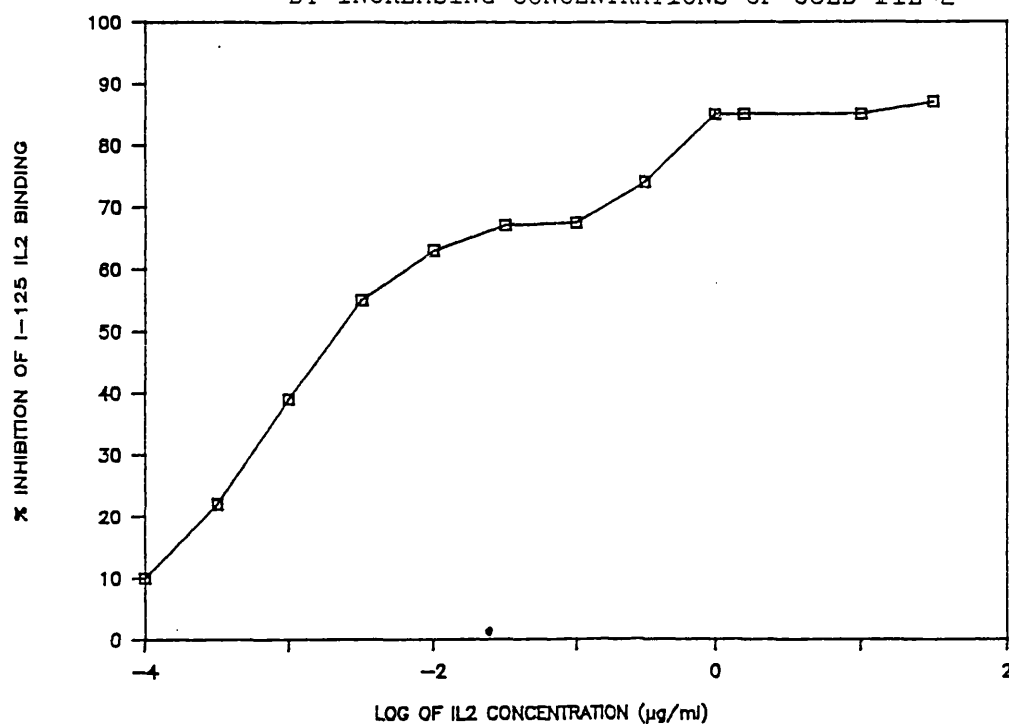
A different approach was therefore adopted to answer the question: does PHMPA treatment inhibit IL-2 binding to its receptor? This work was carried out in collaboration with Mr. Steve Su in the immunology department at Glaxo, where we studied the effect of PHMPA

treatment on the ability of HuT 102/B2 cells to bind rIL-2. HuT 102/B2 cells are unusual, since most long term T and B cell lines of neoplastic and normal origins do not express receptors for IL-2, as assessed by their ability to bind radiolabelled IL-2 [35]. This cell line was used since it is a human T-lymphoma line established from a patient with cutaneous T-cell lymphoma, bearing a high density of high affinity IL-2 receptors and producing IL-2 [36]. Initially experiments were carried out to illustrate the specificity of the binding of ^{125}I -IL-2 to its receptor by showing that increasing concentrations of cold rIL-2 could block this binding (Fig 4.123).

Experiments were carried out to show that the receptor was specific for rIL-2 since neither cold transferrin, nor cold rIL-1, but only cold rIL-2 could block the binding of ^{125}I -IL-2 to its receptor (Fig. 4.124).

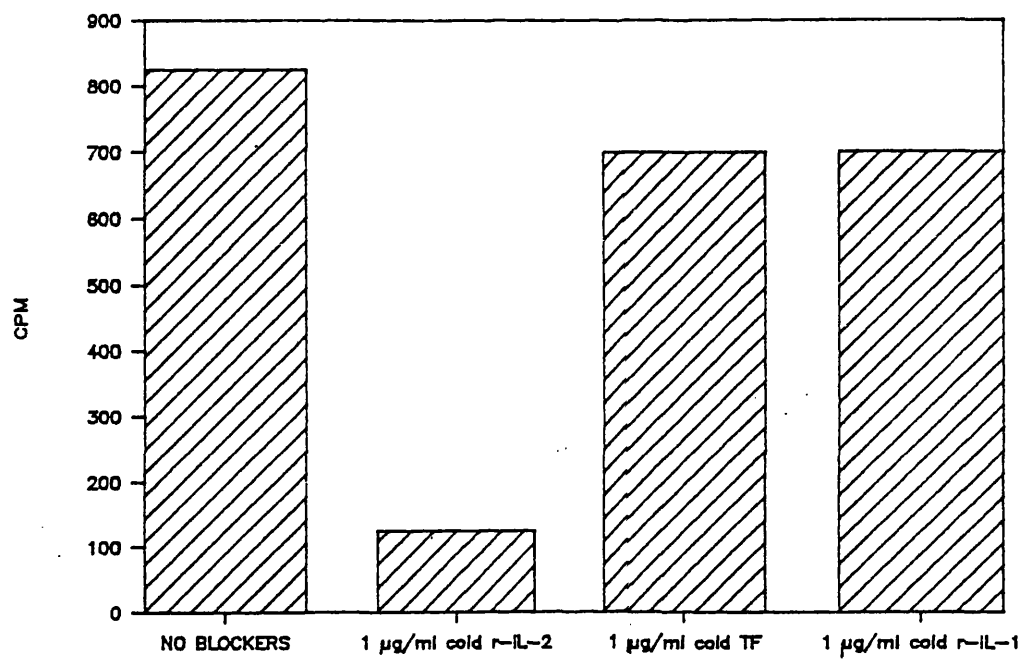
The results of the effect of 1 hour preincubation with 50 μM PHMPA on subsequent ability of HuT cells to bind ^{125}I -IL-2 are illustrated in table 4.36, showing that the average relative percent proliferation was -32% for 3 experiments; however, the data were very variable and most suppression was obtained in experiment 2 using an old label. It is conceivable that the labelled IL-2 used in experiment 2 had in some way become denatured or chemically unstable and lost a significant amount of iodine label (since iodine labelled compounds are known to be more unstable than compounds labelled with other-radioactive isotopes), apart from the fact that CPM's were lower using this batch of IL-2 due to the short half-life of ^{125}I -IL-2. All these reasons make the data from experiment 2 less reliable than those from

FIG. 4.123 INCREASING INHIBITION OF 125 I-IL-2 BINDING TO HUT CELLS
BY INCREASING CONCENTRATIONS OF COLD IL-2



Showing $1 \mu\text{g/ml}$ IL-2 to be an optimal concentration to use in order to maximally inhibit (85%) 125 I-IL-2 binding.

FIG. 4.124 SPECIFICITY OF ^{125}I -IL-2 BINDING TO HUT CELLS



Showing only cold rIL-2 significantly inhibits binding. Cold transferrin (TF) or cold rIL-1 show no significant effect on subsequent ^{125}I -IL-2 binding.

TABLE 4.36 SUMMARY OF RESULTS FROM HuT EXPERIMENTS

EFFECT OF 1 HOUR PREINCUBATION WITH 50 μ M PHMPA ON SUBSEQUENT ABILITY OF HuT-102/B2 CELLS TO BIND 125 I-IL-2. RESULTS ARE EXPRESSED AS MEAN CPM FOR QUADRUPPLICATE WELLS. CPM'S CANNOT BE AVERAGED FROM EXPERIMENTS 1, 2 AND 3 SINCE DIFFERENT BATCHES AND AGES OF 125 I-IL-2 WERE USED WITH THEREFORE DIFFERENT SPECIFIC ACTIVITIES.

TUBE CONTENTS	EXP. No.	TREATMENT AND CPM		RELATIVE % PROLIFERATION VS. NO PHMPSA IN ABSENCE OF r-IL-2	PURPOSE OF EXPERIMENT
		- PHMPSA	+ PHMPSA		
CELLS + 50 μ l RPMI + 50 μ l 125I-IL-2 (5 nCi)	1 2 3	1086 506 1352	971 224 943	- 11 % - 56 % - 30 % $\bar{x} \pm \text{SEM}$ -32 \pm 13 %	TO TEST WHETHER PHMPSA INHIBITS HOT r-IL-2 BINDING TO IL-2-R
CELLS + 50 μ l RPMI CONTAINING r-IL-2 TO GIVE A FINAL CONC. 1 μ g/ml, THEN 50 μ l 125I-IL-2 (5 nCi)		- PHMPSA	RELATIVE % PROLIFERATION VS. NO PHMPSA IN ABSENCE OF r-IL-2		
	1	228	- 79 %		
	2	71	- 86 %		
	3	153	- 89 % $\bar{x} \pm \text{SEM}$ - 85 \pm 3 %		
		+ PHMPSA	RELATIVE % PROLIFERATION VS. NO PHMPSA IN ABSENCE OF r-IL-2	RELATIVE % PROLIFERATION VS. NO PHMPSA + PRESENCE OF r-IL-2	TO TEST WHETHER PHMPSA INHIBITS ABILITY OF COLD' rIL-2 TO BIND TO IL-2-R
	1	199	- 80 %	- 13 %	
	2	58	- 74 %	- 18 %	
	3	129	- 86 % $\bar{x} \pm \text{SEM}$ - 80 \pm 3 %	- 16 % $\bar{x} \pm \text{SEM}$ - 16 \pm 2 %	
NO CELLS ADDED ONLY REAGENTS IN CORRECT ORDER	1	200			BKG COUNTS I.E. REAGENT CONTROL
	2	60			
	3	100			

experiments 1 or 3. The table also shows that rIL-2 substantially inhibits subsequent binding of hot rIL-2, indicating that the label binds specifically to the IL-2 receptor. Not surprisingly in view of its seeming ability to inhibit receptor binding of the hot ligand, PHMPSA also inhibited binding of cold rIL-2; and in fact in the presence of cold or hot rIL-2, binding was slightly more depressed than in the absence of rIL-2.

Work is being continued at Glaxo, to see if by increasing the number of experiments a clearer answer is obtained and also whether similar results are obtained with another cell line IARC 301 and also more relevantly using normal human cells, since it is assumed - but not known for certain - that the high affinity receptor on cell lines and normal PBMNC are in fact identical and behave identically. Certainly the TAC Ag is smaller on the HuT cells - 47-50 kda (P50) [32c] - compared to the human TAC Ag - 52-57 kda (P55) [38-39] - , probably due to different post-translational processing of the β chain.

In the light of these results, it would suggest that inhibition of rIL-2 binding to its receptor could account for some of the inhibition of proliferation seen after PHMPSA treatment of PBMNC and partly why "educated" cells failed to proliferate to rIL-2 after pre or post-treatment etc. with PHMPSA. However, in no HuT experiment was complete inhibition of binding seen, the maximum inhibition being -56%, whereas in proliferation experiments at the same concentration of PHMPSA (50 μ M) 70% to 80% inhibition was seen. It is not known whether this amount of reduction in IL-2 binding could account for the

almost obliteration of proliferation observed; the inhibition of binding of rIL-2 to its receptor could conceivably be because PHMPSA blocks important free SH groups in the active sites of the high affinity receptor.

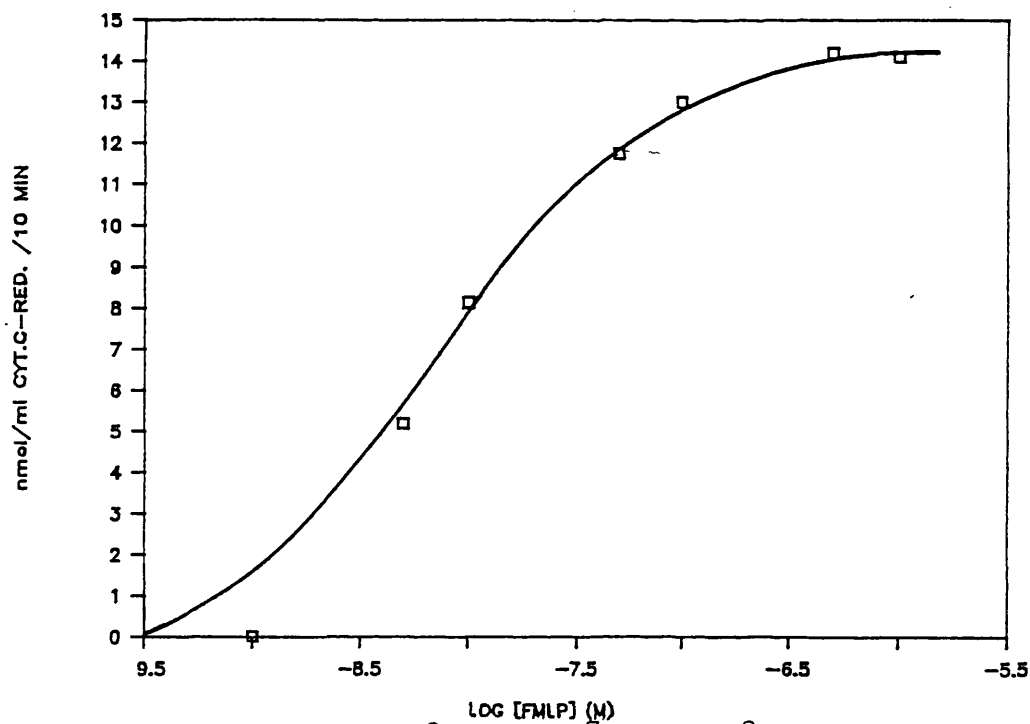
These results still leave open the idea that the main reason for inhibition of proliferation and inhibition of "educated" cells to respond to rIL-2 after PHMPSA treatment is due to an inhibition of signal transduction. Alternatively PHMPSA treatment before mitogen stimulation, or before "education" of cells, could in some way prevent high affinity receptor formation without inhibiting TAC induction. These ideas are further discussed in the following discussion chapter.

4.25. EFFECT OF PHMPSA ON SUPEROXIDE PRODUCTION FROM NEUTROPHILS.

In the light of the results from the HuT experiments, that show a possible inhibition of rIL-2 binding to its receptor after PHMPSA treatment, in another cell system - namely neutrophils - and using two stimuli [one a receptor mediated stimulus (FMLP) [40], the other a non-receptor mediated stimulus (PMA)], at their optimal concentrations shown previously to be 5×10^{-8} M and 10 ng/ml respectively (Figs. 4.125-4.126), in order to investigate superoxide production (Table 4.37), it was found that only receptor mediated superoxide production was PHMPSA sensitive (Table 4.40), similarly to the finding in the HuT experiment. Cell viability was found to be unaffected by 50 μ M PHMPSA treatment (table 4.38) and tables 4.39 and 4.40 show that a 60 minutes incubation with 50 μ M PHMPSA at 37 °C was required to observe this

FIG. 4.125 DOSE-RESPONSE CURVE FOR FMLP (10^{-9} - 10^{-6} M)

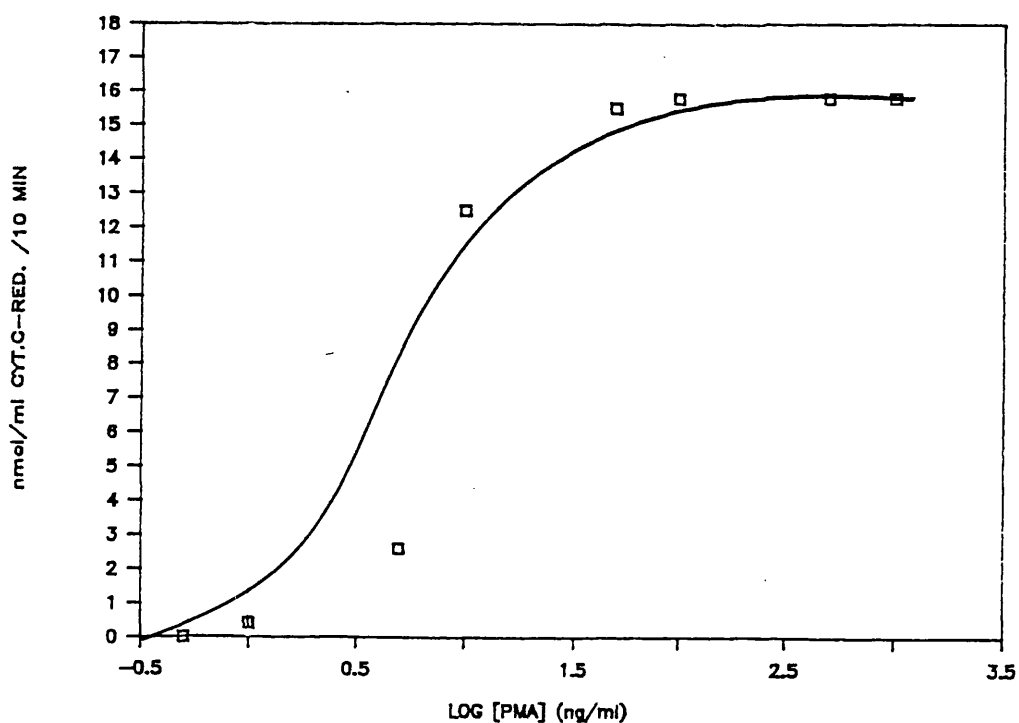
ON SUPEROXIDE PRODUCTION FROM NEUTROPHILS



Optimal conc. was 5×10^{-8} - 5×10^{-7} . 5×10^{-8} M was chosen for all experiments

FIG. 4.126 DOSE-RESPONSE FOR PMA (0.5 - 1000 ng/ml)

ON SUPEROXIDE PRODUCTION FROM NEUTROPHILS



Optimal conc. was in the range 10-20 ng/ml. 10 ng/ml was chosen for all subsequent experiments.

TABLE 4.37

EFFECT OF 2 DIFFERENT STIMULI ON SUPEROXIDE PRODUCTION FROM NEUTROPHILS. RESULTS ARE EXPRESSED AS nmol CYTOCHROME-C REDUCED OVER A 10-MIN. PERIOD ($\bar{x} \pm 1$ SD IN THREE NORMAL SUBJECTS).

UNSTIMULATED	PMA-STIMULATED 10 ng/ml	FMLP-STIMULATED 5 x 10 ⁻⁸ M
1.9 \pm 10.6	15.9 \pm 5.4	12.2 \pm 6.9

TABLE 4.38

NEUTROPHILS VIABILITY STUDIES AFTER VARIOUS TREATMENTS. RESULTS ARE EXPRESSED AS % CELL VIABILITY.

AFTER PERCOLL SEPARATION.....	= 99%
60' AT 37 °C IN ABSENCE PHMPA.....	= 97%
30' AT 37 °C IN PRESENCE OF 50 μ M PHMPA....	= 97%
60' AT 37 °C IN PRESENCE OF 50 μ M PHMPA....	= 97%

TABLE 4.39

EFFECT OF PRETREATMENT WITH 50 μ M PHMPA AT 37 °C FOR 30' AND THEN WASHED OFF, ON ABILITY OF NEUTROPHILS TO BE SUBSEQUENTLY STIMULATED WITH 2 DIFFERENT STIMULI. RESULTS ARE EXPRESSED AS nmol CYTOCHROME-C REDUCED OVER A 10 MIN. PERIOD ($\bar{x} \pm 1$ SD IN TWO NORMAL SUBJECTS, NS = NOT SIGNIFICANT BY PAIRED STUDENT'S T-TEST).

	UNSTIMULATED	PMA-STIMULATED	FMLP-STIMULATED
NO PHMPA	0.00 \pm 0.00	21.25 \pm 4.17	8.74 \pm 0.78
+ PHMPA	0.13 \pm 0.19	18.25 \pm 0.72	9.39 \pm 2.86
		} NS	
		} NS	
		MEAN RELATIVE % O_2^- PRODUCTION	
		- 13%	7%
		(SUPPRESSION)	(ENHANCEMENT)

TABLE 4.40

EFFECT OF PRETREATMENT WITH 50 μ M PHMPA AT 37 °C FOR 60 MIN. AND THEN WASHED OFF, ON ABILITY OF NEUTROPHILS TO BE SUBSEQUENTLY STIMULATED BY TWO DIFFERENT STIMULI. RESULTS ARE EXPRESSED AS nmol CYTOCHROME-C REDUCED OVER A 10-MIN. PERIOD ($\bar{x} \pm 1$ SD IN THREE NORMAL SUBJECTS; NS = NOT SIGNIFICANT, p VALUE BY PAIRED STUDENT'S TEST).

	UNSTIMULATED	PMA-STIMULATED	FMLP-STIMULATED
		10 ng/ml	5 x 10 ⁻⁸ M
NO PHMPA	1.9 \pm 1.6	15.9 \pm 5.4	12.2 \pm 6.9
+ PHMPA	3.8 \pm 0.7	20.9 \pm 6.1	6.3 \pm 2.0
		} NS	
		} p = 0.05	
		MEAN RELATIVE % O_2^- PRODUCTION	
		49%	- 43%
		(ENHANCEMENT)	(SUPPRESSION)

effect since a 30 minute incubation time was too short. It is not known whether the failure for FMLP to stimulate cells after PHMPSA treatment was due to inhibition of FMLP receptor binding as in the HuT experiment, or a failure to transduce a signal as is proposed to be the major cause of failure to proliferate in the rIL-2 stimulated "educated" cells and mitogen stimulated PBMNC assays.

The data for the proliferation experiments, "educated" cells experiments, and HuT experiments can be used to shed light on interpretation about the CON-A induced suppressor assay data and why only "activity" was significantly inhibited by PHMPSA treatment, as further expanded in the discussion chapter.

CHAPTER 5.1 - DISCUSSION

Normal immune responses are controlled by complex interactions of immunoregulatory cells capable of either suppression (termed suppressor cells) or helping (termed helper cells) of both humoral and cell mediated immune responses. All lymphoid cells (T-, B-, adherent-cells of both the polymorphonuclear and monocyte/macrophage series, large granular lymphocytes and NK-cells) seem to show the capacity to act as helper or suppressor cells under different circumstances [1,2]. Suppressor cells can exert their function by several mechanisms either by acting directly on effector cells of a given function, e.g. cytotoxic function, Ig production or delayed type hypersensitivity, or acting on helper T-cells which are themselves capable of influencing the effector cells; alternatively their effects may be mediated by the production of soluble regulatory substances [2].

Disturbances in the balance between effector and regulatory cells and their Ag and Ag-non-specific soluble factors, and amongst the proportion of the regulatory cells themselves is associated with the pathogenesis of various disease states, including models of RA in animals and human RA itself; and as stated in a recent review [3], there is virtually no autoimmune disease in which qualitative and quantitative suppressor T-cell defects have not been reported.

In view of the undisputed immunodysregulation observed in RA resulting in enhanced uncontrolled humoral immunity and reduced

efficiency in cell mediated immunity, and the autoimmune nature of the disease, it was wondered whether defective suppressor cell activity could be a major cause of these defects. Since, whether present as a primary anomaly leading to the development of RA, or present secondary to disease activity (e.g. anti-CD8 lymphocytotoxic Ab), impaired suppressor cell activity could certainly play an important role by allowing the expression of damaging immune reactions against self-components and result in some of the immunological aberrations observed in RA.

RA is also characterized as being an "inflammatory disease" and there is ample evidence for increased phagocytic activity in vivo [4,5,6]. There is also evidence for defective serum and membrane -SH levels [7]. The relationship between these 3 factors was investigated in this project.

Initial experiments reconfirmed the observation of defective serum -SH levels in RA and normal levels in AS/PSA, as observed by others [7]. These results would suggest that inflammation per se does not significantly reduce serum -SH levels, although results from AS/PSA being at the lower end of the normal range indicate a possible cause-effect and suggest that such patients, despite the inflammatory nature of the disease, have the capacity to regenerate reduced -SH groups almost as rapidly as the latter are oxidized. On the contrary, in RA excessive inflammation and a deficiency in the capacity to re-reduce oxidized -SH groups results in this defect. It is interesting that major immune dysfunction is only observed in RA,

suggesting a possible association between inflammation, -SH levels and immunoregulation mediated by cells, since reduced -SH levels would be expected to extend to cell surface membranes and influence cell function.

A sensitive ELISA system was set-up with a useful range for measurement of IgG, extending from 16 to 500 ng/ml, and for IgM 32 to 794 ng/ml. Using such an ELISA system, the necessity for free -SH groups on cell surfaces for correct monocyte-lymphocyte interactions to generate IgG in a PWM-stimulated system was shown by the ability of PHMPSA to inhibit IgG synthesis in a dose-dependent manner with an ID50 of 12 μ M, comparable to figures quoted by others [8,9]. The inhibition was due to surface -SH blockade, and not cytotoxic inhibition, since cell viability was insignificantly affected by PHMPSA treatment, nor by the 1-hour incubation at 37 °C; however, the incubation period did result in a slight loss in ability to produce IgG, possibly due to selective loss of B-cells.

Comparisons of IgG and IgM production in various patient groups and normals revealed that RA-NSAID patients showed defective synthesis of IgG and IgM in response to PWM stimulation, the levels being comparable to those found by others [10,11] and IgG and IgM being of the same order of magnitude - approximately 2000 ng/10⁶ cells - which contrasts with IgM levels found by one author [12], where normal IgM were 5 - 25 times greater than IgG levels. Other authors have reported either significantly depressed [13,14,15] or normal [16,17] or raised [10] mitogen-stimulated IgG production, and several authors found defective mitogen-stimulated IgM production [10,18] possibly

indicating the need to more clearly define RA patients used in investigative studies. This in vitro hyporesponsiveness is suggested to be due to prior activation of the cells in vivo, as evidenced by raised spontaneous IgG synthesis shown in this project. However, raised background IgM synthesis was not observed. Further evidence from other authors for in vivo prior activation of B-cells comes from reports of: 1) spontaneous Ig production from PBMNC and synovial tissues [19]; 2) enhanced spontaneous ^3H -TdR uptake [20,21]; 3) increased number of CD5^+ B cells but fewer able to mouse RBC rosette since prior activated [23,24], the former known to be committed to auto-Ab formation [25]; 4) increased DR expression on circulating B-cells [26] and joint B-cells [27]; 5) spontaneous auto-Ab production, e.g. RF etc.... The hyporesponsiveness to mitogen stimulated Ig production in RA NSAID was not due to altered kinetics of Ig production which was the same in all patient groups compared to normals, and reflects what is happening at the molecular level in the process of B-cell activation. RA patients on the sulphhydrate drug D-Penicillamine showed normal background and stimulated levels of IgG and IgM as did AS/PSA patients, in common with various reports [8,28]. It would have been interesting to have looked at IgA synthesis, since AS/PSA patients are said to show increased synthetic capacity for IgA, and RA and SLE patients deficient IgA synthesis [3], perhaps reflecting a defective suppressor cell system solely for IgA synthesis in the former groups, and excess suppressor cells specific for B-clones making IgA in the latter groups.

Further evidence that surface -SH groups are important for cell

function came from in vitro experiments with 2-ME, showing normalization of defective IgG and IgM synthesis in RA NSAID. 2-ME has long been known to increase proliferation [29] or Ig production [30] and in fact it stimulates Ig production even from normal subjects, as evidenced in this thesis; however, only significant increases were observed in RA NSAID, which is further evidence that it is oxidized membrane -SH causing the defective in vitro responses in RA NSAID.

To parallel the experiments using PHMPSA, which causes -SH blockade by its Hg moiety, experiments were carried out using the most easily available of the physiological oxidizing agents, namely H_2O_2 , and in agreement with other authors [9,31], a slight mitogenic effect of H_2O_2 on IgG production was observed at low concentrations (10 - 50 nM), perhaps by stimulating IL-2 production [32]. At 50 nM to less than 10 μM H_2O_2 , inhibition of IgG was observed in a dose-dependent manner; a 1-hour incubation at 37 °C with 50 μM H_2O_2 caused cell death, said to be due to H_2O_2 in a form of OH^\bullet compromising a multitude of cellular functions in the following order: DNA strand breaks, that cause activation of polyAD-ribose-polymerase, that in turn cause ATP and NAD depletion and an altered redox state, followed by further ATP depletion by stimulation of the hexose monophosphate shunt pathway, Ca^{++} and Na^+ influx and K^+ efflux, then actin polymerization and blebbing of the plasma membrane and finally cell death. Sublethal doses of H_2O_2 cause DNA strand breaks and delayed, but not inhibited, cell division, and therefore abnormal daughter cells [33]. The fact that IgG production (and IgM - data not shown) was affected more by H_2O_2 in the RA NSAID group is perhaps evidence

that the vast majority of their surface -SH groups were already oxidized, thus oxidation of any remaining free surface -SH groups required less H_2O_2 than for the other patient and normal groups.

Two basic approaches can be used to study immunoregulatory cells: 1) phenotypic quantification of such cells; 2) functional analysis of such cells. The former was investigated to some extent, but not dwelt on, due to the many difficulties associated with limited phenotypic analysis, particularly when one only has resources to "single-label" cells. Because of the numerous Mabs now available to subclassify specific functional subsets of cells within the two main immunoregulatory T-cell populations - i.e. $CD4^+$ and $CD8^+$ cells -, a proper phenotypic comparison of immunoregulatory cell balances in RA compared to normals would require multiple labelling techniques, which was not possible to perform. Also, despite a large number of papers that have been published describing changes in T-cell subset ratios or numbers - the implication that such changes are related in some way to the aetiology of the disease in question are frequently unwarranted, since: 1) an increased $CD4^+/CD8^+$ ratio frequently associated with autoimmune disease could mean a fall in $CD8^+$ numbers or a rise in $CD4^+$ numbers; 2) a fall in $CD8^+$ numbers cannot be equated with a fall in T_S numbers, since the $CD8^+$ population contains T_C cells too; perhaps a better method would be to look at T_C/T_S ratios using $CD8^+ CD11^- CD28^+$ and $CD8^+ CD11^+ CD28^-$ respectively; 3) a rise in $CD4^+$ numbers cannot be equated with a rise in T_H numbers, since the $CD4^+$ population contains $T_{H/I}$ and $T_{S/I}$ cells, thus a better method would be to look at these ratios; 4) findings from the blood are not necessarily representative

of an individual "in toto"; 5) if suppressor cell dysfunction is a contributing mechanism to autoimmunity, the restrictive nature of most auto-Ab would require a selective rather than overall loss of T_S numbers and/or functions and this would not be expected to result in detectable changes in $CD4^+/CD8^+$ ratios in the blood; 6) changes in subset ratios may reflect compensatory responses of the host to chronic Ag stimulations rather than intrinsic immunological defects; 7) subset ratio changes do not necessarily result in immunodefects: e.g. in chronic neutropenia $T_{S/C}$ numbers are raised with few clinical problems; 8) a normal ratio may hide underlying imbalances since both the $CD4^+$ and $CD8^+$ subsets are heterogenous populations; 9) one needs to support phenotypic ratio changes with functional assays relative to the disease under question; 10) phenotypic analyses rely on the assumption that marker-function relationships that have been established in healthy subjects hold true during disease, and this may not be so: e.g. do $CD8^+$ cells said to be $T_{S/C}$ in health have the same function in RA? Thus, $CD8^+/CD4^+$ ratios probably have little or no diagnostic or prognostic value clinically.

Thus, a more functional approach was preferred for this project. In vitro models to investigate suppressor cell activity in man are complex and still at a developmental stage but of the possible models available the methods elected to be used in this project were: CON-A inducible suppressor cell activity assessing successive suppressor cell generation by suppression of IgG or IgM synthesis, or mitogen stimulated T-cell proliferation and short-lived suppressor cell activity which probably measures spontaneous suppressor cells.

Several papers have been written on multifactor studies of the CON-A suppressor cell assay [34] and it would seem that the system is largely controlled by the interactions between different environmental variables, those in the CON-A suppressor cell generating system including: 1) the concentration of CON-A; 2) length of culture period; 3) time of addition of CON-A; 4) cell concentration; 5) the length of contact period between cells and CON-A. In the indicator co-culture system the variables are: 1) relative ratio of responder/effector cells; 2) total cell concentration; 3) mitomycin-C treatment of effector cells; 4) whether CON-A was washed off the putative suppressor cells or not; 5) length of culture period; 6) time of addition of CON-A treated cells to the responder cells; 7) the type of serum in medium affecting background responses (ISCOVE, autologous serum, low mitogenic FCS and bovine serum-albumin, giving lower backgrounds than mitogenic FCS and AB serum); 8) whether results are expressed relative to untreated co-culture cells or responder cells alone; 9) as pointed out by Gershon, the effect that the regulatory cells produce depend also on the activity of the responder cells - which he called the "second law of thymodynamics"! Thus, the exact condition used in either culture system and the way the results are expressed determines the quantitative levels of suppression observed.

Therefore, in this project a multifactor study of environmental variables in both the CON-A suppressor cell generating system and indicator co-culture system, that might influence optimal suppression, was carried out using PBMNC from normal subjects. An optimal concentration of CON-A was found to be 10 $\mu\text{g/ml}$ for both IgM and IgG

assays, the finding that the same concentration was optimal for both was also observed by others [35] who looked at IgG, IgM and IgA production. Interestingly a wide range of CON-A concentrations obtained from a number of sources can activate suppressor activity, various laboratories have used $1 - 100 \mu\text{g}/10^6$ cells and while it is not clear if the same suppressor cell is activated in each case, it has been reported that doses used to produce a strong mitogenic response are not necessary for suppressor cell activation. Interestingly, $10 \mu\text{g}/\text{ml}$ was optimally mitogenic in this thesis, and another laboratory found an optimal dose to be mitogenic [36]. Mitogenically superoptimal concentrations were found to give greater suppression, e.g. $25 \mu\text{g}/\text{ml}$, but $10 \mu\text{g}/\text{ml}$ was chosen as the stimulating concentration for generation of suppressor cells, since this concentration gave near-maximal suppression of Ig synthesis (about 90%), and unlike $\geq 25 \mu\text{g}/\text{ml}$, this concentration did not affect cell viability or cell yield when compared to untreated cells. A suppression of approximately 80% has also been achieved using this assay for suppression of B-cell responses, e.g. PFC in autologous cultures by others [36]. It was observed that very low doses of CON-A ($0.05 - 1 \mu\text{g}/\text{ml}$) sometimes resulted in enhancement rather than subsequent suppression of PWM stimulated Ig synthesis. This observation has also been noted by Gupta et al. [35]: in their experiments they found that low doses of CON-A ($0 - 5 \mu\text{g}/\text{ml}$) caused a rise in $T\mu$ cell numbers, and $10 - 40 \mu\text{g}/\text{ml}$ a fall, but that numbers of $T\gamma$ cells rose steadily $0 - 80 \mu\text{g}/\text{ml}$; they suggested that, depending on the dose of CON-A used, helper cell induction ($0 - 5 \mu\text{g}/\text{ml}$) or suppressor cell induction ($10 - 80 \mu\text{g}/\text{ml}$) could occur. The induced

suppressor cell activity was associated with an increase in T_γ cells and , since it is agreed that most T_S cells for suppression of PWM stimulated Ig synthesis are also T_γ cells, this would agree with observations in this project, of increased CD8 expression with increasing doses of CON-A. This would indicate that the expression of the CD8 Ag seems to be associated with suppressor function; however it must be remembered that, due to functional and morphological heterogeneity within a group of cells detected using a particular Mab, it is essential to define the specificity of the Mab and its cross-reactiveness. The CD8 Ag, for instance, appears on suppressor T-lymphocytes, cytotoxic lymphocytes and NK-cells, the latter two can also function in a suppressive way, but it is via a cytotoxic mechanism; since there is no evidence of significantly reduced cell viability in the CON-A co-culture a lytic mechanism does not seem to be responsible for the suppression observed. It would seem that CON-A treatment induces cells that act functionally as suppressor cells and the majority of which appear to be of the $CD8^+$ phenotype. To further specify the exact phenotypic nature of the induced suppressor cells, Mabs to functionally unique monocytes, B-cells, subsets of $CD4^+$ cells and subsets of $CD8^+$ cells should be used. Thus, defining the actual suppressor cell or mixture of cells induced is very complex, due to the functional and phenotypic heterogeneity which exists in all lymphoid cell subsets. The inability of complement and CD8 Ab to abolish the induced suppressor activity would suggest the co-existence of non- $CD8^+$ suppressor cells (e.g. perhaps $T_{S/I}$ or even non-T-cells. Gupta et al. [35] suggest in unpublished observations the existence of a partial correlation between alterations in T_γ -cell subset

proportions and in vitro suppressor cell activity for B-cell differentiation. The lack of an absolute correlation is most likely attributable to the fact that there are at least two subpopulations of $T\gamma$ -cells, and only one of them seems to act as a true suppressor population. Gupta also suggested that the increase in $T\gamma$ -cells could be due to induction of $T\gamma$ -cells from a population of T-cells with neither IgM nor IgG receptors, or by causing $T\mu$ -cells to differentiate to $T\gamma$ -cells by receptor switching - that he found to occur at high CON-A concentrations. In support of this, two reports [37,38] found that $T\mu$ - and non- $T\gamma$ -cells pulsed with CON-A could suppress plasma cell generation or PFC formation, suggesting the existence of at least two functionally distinct $T\mu$ -cells, with helper/inducer and helper/suppressor properties; in support of this it has been shown that separate populations of $CD4^+$ and $CD8^+$ cells can be induced by CON-A to suppress co-cultures [39,40], and yet another group using negative panning found CON-A inducible suppressor activity in the $CD4^+$ population [41], and it has been shown that $T_{S/I}$ can themselves cause suppression of polyclonal Ig production [42] and that anti-CD45R Mab can prevent $T_{S/I}$ function in the generation of suppressor cells in AMLR and in CON-A induced suppressor activity [43]. Perhaps this explains the observation in this project of the inability of complement and anti-CD8 Mab to totally abolish suppressor activity, particularly in view of the fact that non-T non-induced suppressor cells also coexist with CON-A induced suppressor cells. However, Reinherz [44] believes that suppressor function is only present within $CD8^+$ populations, although his experimental design could be criticised, since he used positive panning - which is known to prevent

proliferation in autologous or allogeneic MLR [45] - thus CON-A would not be expected to have been able to stimulate suppressor activity in the $CD4^+$ population, due to methodological artifacts.

In agreement with recommendations [46], a 1:1 ratio of effector to responder cells was found to be optimal for observing consistent and maximal suppressor activity, as found in most reports [12,36]. Three approaches were adopted to find the optimal ratio: 1) varying the ratios but keeping the total volume the same, using this method ratios of 1:3 up to 3:1 showed increasing suppression, however 1:1 to 3:1 were not significantly different; 2) varying the number of effector cells added to a constant number of responder cells where total volume altered, using this method suppression was found to increase when the ratio was altered from 1:2 to 1:1, but declined from 1:1 to 3:1; 3) varying the ratios of effector cells to a constant number of responder cells keeping the total volume the same, suppression was with this method found to steadily decline from 1:1 to as low as 1:4000; but, if good suppression is taken as > 75%, this was only still obtainable up to a ratio of 1:10. Other authors using peripheral blood lymphocytes and autologous B- and T-responses found increased suppression as the ratio of effector/responder cells was lowered from 1:10 to 1:1 [47], and others, using synovial fluid lymphocytes and allogeneic T-cell responses, as the ratio was raised 1:2 to 2:1 [54]. In agreement with most authors, the 1:1 ratio consisted of 2×10^5 suppressor cells added to 2×10^5 responder cell in a 200 μ l volume, the other popular cell density is 1×10^5 of each cell type per 200 μ l. Another report found suppressor activity

increased as effector/responder ratio for tonsil lymphocytes were reduced from 1:30 to 1:1, and no suppressor activity occurred at 1:300. The same author found suppressor activity using peripheral blood lymphocytes increased from a 1:10 to 1:1 ratio [36]. This author looking at CON-A induced suppression of PWM stimulated PFC of autologous cells found only suppressor activity, even when low numbers of suppressor cells were added, which is in agreement with results in this project and agrees with results of another author [48], but contrasts to findings by others [49;50]. The findings in this project support the concept that the suppressor cells induced are functionally and physically (a) distinct population(s) which mediate only suppression. It appears that these cells are not capable of switching function (i.e. helper \longleftrightarrow suppressor) depending on either the strength of the stimulus or the relative number of such cells. This is in agreement with current evidence indicating that help and suppression are mediated by distinct subpopulations of lymphoid cells.

Various papers suggest that suboptimal concentrations of mitogen for observing suppression of T-cells autologous responses are preferable in the indicator co-culture, since they suggest that: a) suppression is more difficult to quantitate using optimal doses; and b) suboptimal doses provide a more reliable index of inhibitory effects [34]; c) it enhances the sensitivity of the assay, particularly if CON-A is used in suboptimal doses too [47]. Another author suggested that one should not look at differences at individual PWM doses, but compare the area under dose-response curves [51]. However, a 1:200 PWM dilution (optimal for Ig synthesis) was found to

be optimal for the expression of maximum suppression in this project. In contrast to these former views, it might be mathematically easier and more accurate to express relative percent Ig synthesis relative to untreated co-cultures in which the responder cells are showing particularly good responses to PWM in terms of Ig synthesis, as has been suggested in one report [52]. It was wondered whether increasing the co-culture period to 10 - 12 days (so that Ig production would be at its peak) would lead to improved suppression. This was found not to be the case. Alternatively, the cultures could have been grown in U-shaped as opposed to □-shaped wells, since it was found that vessel architecture has a profound effect on subsequent Ig production, which was found to increase when cells were grown in U-shaped vessels compared to □-shaped (data not shown). This was probably due to the fact that such a shape provides an optimal surface for maximum contact between cells (B-cells, T_H , T_S , monocytes, macrophages, etc.) and also minimum distances for lymphokine transfer between cells and therefore optimal conditions for maximum Ig production. However, because very good suppression was obtained using a 7-day culture and □-shaped wells, these possibilities were not pursued.

Another author [34] found pre-culturing cells for 24 hours resulted in a reduction in subsequent mitogenic responses, but a 5-day pre-culture resulted in a loss of spontaneous suppressor cells, so that such cells responded in an enhanced manner compared to fresh cells when subsequently stimulated with mitogens. Such precultured cells contained few B- and T_S -cells but many T_H cells, however sufficient B-cells for the T_H to stimulate on addition of mitogens. In

the light of Dixon's [52] argument that better suppression is observed in indicator cultures where responder cells are showing strong immune responses, it might have been expected that, if responder cells were grown for a long pre-culture period sufficient to allow them to escape from an inhibitory mechanism (spontaneous suppressor cells), a better suppression might have been observed on addition of putative suppressor cells than when fresh responder cells are added. However, no advantage using this method was observed [34].

Thus, a 1:200 PWM and U-shaped wells and fresh autologous responder cells were used in the indicator system in this project. PWM is a polyclonal T-dependent B-cell activator, thus the advantage of this mitogen over T-independent mitogens is that the resultant effect reflects helper and suppressor signals generated by T-cells, the interaction of these signals with B-cells and the capacity of B-cells to produce and secrete Ig. Thus, Ig production reflects overall T-regulator and B-effector capabilities and not just B-cell function per se - which is what T-independent mitogens do. One problem using PWM is that 10% - 12% of the general population do not respond to it, and some people respond sometimes but not other times, although most workers agree that low responders are consistently low and high responders are consistently high, and people in-between represent a heterogeneous group [53]. It was confirmed in this project that certain individuals do not respond to PWM, and no meaningful data could be obtained from such subjects: other CON-A induced suppressor cell experiments were abandoned because of lack of response to CON-A also observed by other investigators [54,39], however this was a rare

problem. To decipher which mechanism was responsible for a lack of effect, wherever possible responder cells were set-up with or without PWM alone, to check that the defect was not simply due to non response to PWM.

One of the practical difficulties in this assay is that it requires two bleedings from the same person, which can be a problem with patient material, in particular RA, since such patients tend to be anaemic and require frequent bleeding for other routine tests. It has been stated that one can use unstimulated cells grown in pre-culture for 48 hours as a source of autologous cells, but this appears only suitable for looking at suppression of a proliferative assay [55], using either autologous or allogeneic suppressor cells [34]. In this project, because of the selective loss of B-cells in the untreated cell populations as noted by Dwyer [34], such a population is unsuitable as a responder population when looking at Ig synthesis. In two normals another method of obtaining suitable responder cells from one bleed was investigated, in which cells were suspended for 48 hours at 4 °C in HEPES buffer containing 10% FCS in a small flask. These cells proved suitable responder population for one person but not for the second (data not shown), thus this method was abandoned despite its successful use by others [28]. A third possibility to avoid a second bleed was to use PBMNC from a different individual as responder cells, such cells could be placed in medium containing 10% DMSO and aliquoted and stored into liquid nitrogen until needed, as used successfully in one report [55]. However, then one is looking at an allogeneic indicator system which becomes more complicated to

interpret. Thus, despite its drawback, an autologous responder system using freshly prepared cells was the method of choice in this project.

More recently, two authors have suggested improved versions of the CON-A induced suppressor assay. Panush [56] suggested that to avoid such problems as a second bleed for autologous responder cells, or finding willing donors for allogeneic responder cells (which can vary in their responsiveness), or the frequent manipulations of cells on 2 different days and frequent adjustments to correct for loss of cell viability, or the time-consuming tedious incubations and washing of putative suppressor cells with α -methyl-mannoside, - one can use an IM9 lymphoblastoid B-cell line as the responder cell population. Such cells are easy to grow, readily available, not affected by CON-A present on effector cells, they eliminate the problem of MLC responses and can be assayed quickly, thus providing a reproducible standardized accessible source of responder cells with which to compare suppressor activity. Panush advocated mitomycin-C treatment of PBMNC before stimulation with CON-A, placing them in a microtiter plate for a 48-hour culture period with CON-A, then directly adding an equal number of IM9 cells to the well and culturing for a further 72 hours to determine the percentage suppression of ^3H -TdR uptake or IgG secretion by these IM9 cells. Panush found this method to be comparable to the classically used methodology. He found that other possible cell lines that could be used included RAJI, MOLT-4 and BJAB. Two other authors have found cell lines to be suitable responder cells [57,58]. A second alteration in methodology was described by Knaab [59], who stored cells to act as responder cells and used only a

16-hour pre-culture with CON-A.

CON-A appears to cross-link cell surface receptors in activating suppressor cells [60]. CON-A binds to non-T, T and B lymphocytes, thus it is theoretically possible that despite extensive cell washing with culture medium, CON-A will be carried over into the indicator system. One report measured 1% carry-over by ^3H -labelled CON-A [56]. Most examinations of this possibility [34,47], but not all [61], have concluded that in practice this does not occur to any significant extent. However, because it is a possibility and could interfere with result interpretations, it can easily be avoided by the use of a washing stage with α -methyl-mannoside which displaces the CON-A. One report showed that, if CON-A is not washed off when using a T-cell proliferating indicator system, the bound CON-A caused increased proliferation of responder cells and reduced suppressor activity [61]. In yet another report, using a B-cell indicator system, it was shown that bound CON-A caused a dose-dependent suppression of a PWM stimulated PFC response, this suppression was maximal at the concentration of CON-A found to be optimal for generation of suppressor cells [36]. These authors found that if α -methyl-mannoside was added together with CON-A in a 48-hour pre-culture, no suppressor cells were produced. To check that the suppressive effects of the CON-A generated suppressor cells was not due to carry-over of CON-A on such cells, but due to functional suppressor activity of such cells, they added α -methyl-mannoside directly to the co-culture and showed that this did not inhibit subsequent suppression of Ig synthesis. The same authors showed that after 0.3 M α -methyl-mannoside treatment no

CON-A was bound, since a fluoresceine-conjugated fragment of rabbit anti-CON-A Ab showed-up no surface fluorescence [36].

It has been suggested that even after α -methyl-mannoside treatment, 10^6 CON-A generated suppressor cells would carry-over 30 - 40 ng CON-A to the co-culture. This amount is not suppressive when added directly to PWM stimulated cultures, thus 0.3 M α -methyl-mannoside treatment is effective at washing surface bound CON-A [36,53]. In this project washing at room temperature with 0.3 M α -methyl-mannoside was found to be optimal, since although usually equally good, in some initial experiments, 3 mM treatment was found to be inadequate. Other workers prefer to treat the CON-A generated effector cells with 0.3 M α -methyl-mannoside for 30 minutes at 37 °C [41], or at room temperature for 20 minutes with α -methyl-mannoside [61]. Because there is a slight carry-over even after α -methyl-mannoside treatment, some authors choose to "pulse" the untreated cells with a similar dose of CON-A to that used to generate suppressor cells, to control for the effect of CON-A itself carried-over, before both populations are treated with α -methyl-mannoside [36]. In this way, the untreated population is a better sham suppressor population since it removes the possibility that suppression is due to CON-A itself bound to the cell surface, rather than due to the function of CON-A induced cells themselves. Pulsing is only carried out for a few minutes, which is insufficient time to create suppressor cell activity in the untreated cell population, since a minimum of 24 - 48 hours is necessary for optimal suppressor cell activity [61]. This however was not carried out in

this project. Finally, the use of CON-A in solid form - e.g. bound to sepharose beads - can minimize carry-over, but since in this form it could stimulate B-cells to express suppressor activity [53,60], this was not used. However, soluble CON-A can stimulate B-cells to proliferate, but the cells do not become suppressive [47].

It was found that the CON-A induced suppressor cell assay, where successful generation of suppressor activity was achieved, as assessed in a PWM stimulated IgG producing system, was highly reproducible within one individual, and few normal individuals were found who did not respond suppressively.

However, using a mitogen-stimulated or MLC indicator culture (which undoubtedly detects different T_S subsets to that measured in assays of B-cells responses) [1], it has been stated that 12 - 15% of apparently healthy normal subjects cannot be stimulated by CON-A to induce suppressor cells [1,39]. It has been stated that failure to develop suppressor activity could be related to high and low responder status of TH_2^- cells (detected by heteroantisera), which influences suppressor cells [1]. If so, one would expect such a status to be consistent in one individual; however, Dwyer [34] did not find any normal individual who consistently failed to develop suppressor activity after stimulation with CON-A. In fact, it seems more likely that variations per individual were due to cyclical changes in the responsiveness of cells exposed to CON-A, so that not everyone would respond to CON-A at any one time and individuals may respond on some days and not others. Simultaneously comparing suppressor cell activity generated by CON-A in both 3- and 4-day culture, Dwyer found no normal

subjects who did not express good suppressor cell activity on one of the two days. This is of considerable importance if the assay is to be applied to clinical studies, particularly in RA, where failure to respond to CON-A could erroneously be attributed to disease. This is one of the reasons why a B-cell response was investigated, since it seems more reproducible. Another cause for an apparent lack of CON-A induced suppressor activity in certain individuals could be due to spontaneous suppressor activity in untreated co-cultures, this would result in poor suppressor activity in CON-A treated co-cultures, since suppression is calculated as a ratio of the latter to the former (Dwyer and Johnson, unpublished observation). Haynes et al. [53], using allogeneic co-cultures, also found that in certain individuals the untreated cells cause stimulation of responder cells, and in others suppression. They found that repeat co-cultures of untreated cells from subjects whose cells originally suppressed, demonstrated subsequent consistent suppression of PWM induced PFC responses in allogeneic co-cultures. This correlated with non-responsiveness of the donor of the untreated cells to PWM induced PFC. Thus, a non-responsive state to PWM was associated with the presence of naturally occurring suppressor cells, which spontaneously suppress PFC responses to the same extent as did the CON-A induced suppressor cells from donors who responded normally to PWM. The non-responsiveness to PFC after PWM stimulation was not due to an inability of PWM to stimulate the cells, since blastogenic response were equal to those in normal responders, but was solely due to spontaneous suppressor cells, which do not need a 24-hour pre-culture for their induction. Thus, 88% of normals are responsive to PWM in a PWM induced anti-SRBC PFC assay

system, and 12% non responsive due to contaminating radiation-sensitive spontaneous suppressor cells, which are known to occur naturally in 10% of normals; normals show cyclical responsiveness and unresponsiveness to PWM, so that they do not remain poor responders [53]. Likewise, Schwartz et al. [62] also found untreated cells suppressed PWM induced IgG synthesis of autologous fresh PB cells, but unlike the above report where this happened in only 12% of normals, they found it to occur in all co-cultures.

In this thesis putative suppressor cells and untreated cells were removed from the pre-culture by agitation with a rubber policeman and subsequent aspiration with a glass pipette, as is recommended [46].

The nature of the CON-A generated suppressor cells and the nature of the spontaneously occurring suppressor cells that are sometimes detected by this assay are still in debate. Certainly, it seems that CON-A induces a heterogeneous subpopulation of cells to suppress within given systems and in different systems [37,47,69]; dependent on the concentration of CON-A used and the indicator system used to monitor them. However, most reports agree that the major suppressor cell that is triggered by CON-A is a thymus-derived lymphocyte, mainly of the $CD8^+$ phenotype. Thus different subpopulations are involved in suppression of PWM stimulated Ig synthesis compared to suppression of MLC or mitogen stimulated T-cell proliferative responses. Therefore, information gained from suppression of one indicator system may not apply to other systems. Thus there is a need for careful description of assay conditions in order to be confident that the same suppressor cells performing the same functions are being examined. For instance,

as reviewed by Dwyer [1], 1) CON-A induced suppressor T-cells that inhibit Ig production from B-cells are said to be mainly $T\gamma^+$, steroid sensitive, radiation-sensitive and theophylline positive; 2) CON-A induced T_S cells that inhibit mitogen stimulated proliferation are present in the $T\mu$ and $T\gamma$ populations, are histamine receptor positive and steroid resistant - but their precursors are steroid sensitive - ; 3) T_S cells that inhibit allogeneic MLC are mainly TH_2^+ and most of these cells are cytotoxic, they are steroid resistant and radiation insensitive - however their precursors are non-adherent, short-lived and slightly radiation sensitive -; 4) T-cells that inhibit autologous MLR are radiation insensitive and steroid resistant. CON-A suppressor cells are primarily found in $CD8^+ CD11^-$ subsets which respond better to CON-A than $CD8^+ CD11^+$ cells [63]. Spontaneous occurring suppressor cells that are sometimes detected in this assay seem to exist in the form of T- non-T- and adherent-cells [53], and may include radiation-sensitive PGE_2 stimulated suppressor cells that regulate IL-2 production [64,65].

It was decided to express the suppressor data using a formula that would result in negative percentages if suppression has occurred. Similar formulae have been used by numerous other workers [54,66-68]. Results were expressed in the classical way in terms of CON-A co-cultures relative to untreated co-cultures. However, some workers have found that results can be expressed relative to fresh cells when insufficient untreated cells are available [69]. Some authors prefer this method, [62,70] since they have found that untreated cells themselves suppress IgG production of responder cells, however not

significantly like CON-A treated cells do, and they feel that expression of suppressor activity relative to untreated co-cultures could thus lead to false positive results for reduced suppressor activity. This would suggest the existence of spontaneous suppressor cells in the untreated population, as also observed by Haynes [53].

It was shown that the only Ig producing cells in the co-cultures were the responder cells. Occasionally, individuals were found in whom the effector cells (untreated or CON-A treated), when stimulated with PWM alone, did produce Ig; Ig production was however insignificant compared to that from fresh responder cells not pre-incubated for 48 hours (personal observation). This is in agreement with data by Haynes [36,53] who found a non-existent or low plaque forming response (which is a reflection of Ig production) in CON-A or untreated cells set up in culture alone. One might have expected that cells grown in culture with CON-A might not be able to be stimulated to produce Ig by PWM, since any suppressor cells generated within this population would suppress responses of any remaining B-cells within the population. However, to explain the lack of response to PWM from untreated cells alone different reasoning is required. It could be argued that there may be preferential loss of B-cells during the 48 hour pre-culture. This is reasonable, a) since it is known that they can easily adhere to plastic; and b) since Dwyer [34] - who studied losses of lymphocytes in culture - found that many die within the first 24 hours of culture and then more slowly; he found that after 3 days there was a 70% loss of original T-cells and 100% loss of B-cells if no mitogen was present. Thus the untreated population will certainly represent a

different population of cells to fresh cells. This is another reason why some workers advocate the use of fresh cells only for comparative purposes.

In agreement with other reports [53], only CON-A treated effector cells significantly suppressed Ig synthesis from responder cells in the co-culture, whereas the untreated cells did not. In a few experiments it was observed that untreated cells either enhanced (probably due to loss of short-lived suppressor cells) [53,71,72] or suppressed (probably due to spontaneous suppressor activity) Ig synthesis from responder cells. In the former case a suppressor activity would be overestimated, and in the second underestimated.

Because of known differences in potency between different batches of CON-A and PWM, the same batches were used throughout the experiments.

Combining the results gained from this project and the literature, CON-A induced suppressor activity of T- and B-cells responses has been shown to be not the result of the following artifactual explanations for the inhibitory phenomena observed: 1) reduced cell yield; 2) reduced cell viability of responder cells, since viabilities in untreated and CON-A co-cultures were similar, i.e. suppression occurs via a cytostatic mechanism, not a cytotoxic one. It should be remembered however that there could be selective killing of a specific population of T_H cells responsible for the immune response, whose numbers are relatively small and therefore not noticeable in cell viability studies; 3) binding and/or inactivation of

mitogen used in co-culture; 4) exhaustion of in vitro nutrients or overcrowding, since suppression was still apparent at cell densities far below those at which these possibilities could represent a problem (Fig. 4.27); 5) CON-A carry-over; 6) thymidine degradation in proliferative indicator systems; 7) cold thymidine production from macrophages or monocytes which could inhibit DNA synthesis and proliferation in proliferative indicator systems; 8) dead or dying suppressor cells releasing toxic substances which could inhibit responses of responder cells; 9) altered responder kinetics; 10) catabolism of IgG; 11) nor as a result of aggregation of CON-A induced suppressor cells, since these cells were resuspended before being placed in co-culture.

However, there is still controversy in the literature over yet at least another 5 possible artifacts that could explain the inhibitory phenomena of the so-called MHC unrestricted Ag-non-specific CON-A induced suppressor activity:

1) Of most interest with respect to this project is that suppression may result from removal or consumption of essential nutrients, e.g. IL-2 by CD25⁺ suppressor cells; this argument has been applied not only as an explanation for suppression observed in mitogen or allo-Ag stimulated proliferative systems, but ^{to} also account for PWM stimulated Ab producing systems [73,74] and for suppression of MLR [75]; this is said to remain a likely possibility in the light of experiments showing PHA activated cells or RAJI blasts [59] are able to suppress the stimulation of responder cells. Further evidence for this argument is that CON-A activated T-cells, that are pre-treated with IL-2, lose their suppressive abilities [73], and that

cyclosporin-A - known to inhibit the expression of IL-2 receptors - inhibited suppressor activity determined in both PHA or allo-Ag induced proliferative assays and PWM induced IgG synthesis assays [73], and addition of IL-2 abrogates the suppression seen [75]; however it is an unlikely explanation where suppression of certain proliferative responses is being examined, for example when mitomycin-C treated suppressor cells are added to mitogen stimulated auto- or allogeneic cells or MLC, since such suppressor cells are in fact not proliferating (or only poorly), so they would not be binding enough IL-2 to warrant this explanation [59]. Removal or consumption of IL-2 by CD25⁺ suppressor cells could however be a possibility in assays such as the one in this project, where B-cell synthetic responses are observed, since the putative suppressor cells are not mitomycin-C treated and therefore not inhibited from proliferating, thus they could conceivably bind and consume large amounts of IL-2.

2) It has been suggested that this assay does not measure suppression, but "relative ability to provide help", since in proliferative autologous indicator systems the level of suppression has been found to be dependent on background counts [76]. In other words, high background counts may cause artifactually low suppression. This particular artifact can be removed by using low mitogenic FCS, AB serum, or better still serum-free ISCOVES medium [77]. The same authors found that responder cells were stimulated by mitomycin-C treated CON-A cells even after CON-A removal by α -methyl-mannoside and in the absence of mitogen. Whether this was due to residual CON-A or altered suppressor cells membranes making them mitogenic, is not known. However, as long as the stimulation is less than that by

untreated cells, since results are conventionally expressed relative to those in untreated co-cultures, suppression would be observed. This stimulation by CON-A treated cells is not usually reported, since backgrounds are usually subtracted before calculating suppressor activity. The authors therefore suggest that the conventional way of expressing suppression is inadequate and possibly misleading.

3) The fact that CON-A activated spleen cells suppressed the primary in vitro immune response of syngeneic spleen cells to SRBC, but enhanced the in vivo anti-SRBC responses when transferred to syngeneic untreated mice, suggests that lymphocytes acting suppressively in vitro may be helper cells in vivo, and that suppression in vitro is therefore an artifact. Alternatively, the two functions may be mediated by two distinct cell subsets [78].

4) There could be exaggerated helper effects in control cultures (due to loss of SLSA), since no suppression is sometimes observed if results are expressed relative to fresh cells alone [53,71,72].

5) Defective CON-A induced suppressor activity may reflect any of the following, rather than a defect in the CON-A induced suppressor cells themselves: a) excess spontaneous suppressor activity [53,62,70]; b) a blocked or faulty second receptor site for CON-A to stimulate suppressor activity [39] - despite a normal site for blastogenic transformation -, because some normals proliferate well with CON-A but show poor CON-A induced suppressor activity; c) excess non-suppressor T-cells (e.g. excess $CD4^+$ cells) which dilute out the T_S cells; d) defective responses of T-, B- or monocytes to suppressive signals; e) lack of suppression of MLC due to the HLA-D locus on

allogeneic cells being too alike that on autologous cells, so that there is no reaction to suppress; f) excess contrasuppressor cells amongst TH_2^- ($CD4^+ CD8^- CD3^+$) helper cells [1,79] or amongst $CD8^+$ cells [80], that inhibit suppressor cells.

The cellular and soluble factors (Ag-specific and/or MHC-restricted and non-Ag specific) required for Ag-non specific suppressor T-cell activation, growth and differentiation remain poorly understood and appear complex. They seem to vary depending on the suppressor cells in question. For example, for optimal CON-A induced suppression of PWM stimulated Ig synthesis by $CD8^+$ cells require $CD4^+$ $HLA-DR^+$ (probably $T_{S/I}$) to supply important regulatory lymphokines. For optimal CON-A induced suppression of MLR by $CD8^+$ cells, $CD4^+$ $HLA-DR^+$ and $CD8^+$ $Leu-8^+$ and $CD8^+$ $Leu-8^-$ cells are needed. For optimal suppression of PHA stimulated proliferation, $CD8^+$ cells require low density (activated) macrophages, probably in order to supply IL-1 and/or fatty acid products (e.g. PGE_2) [1]. For optimal PWM induced suppression of PWM stimulated $CD4^+$ cell proliferation by $CD8^+$ $CD11^+$ $CD28^-$ cells, the following conditions are required: the $CD8^+$ cells must be activated via PWM stimulation of the T-cell receptor by monocyctic Ag-presenting-cells. $T_{S/I}$ cells must supply T_S growth factor and 8,000 MW lymphokine [81] to stimulate IL-2 receptor expression and later produce IL-2 to stimulate $CD8^+$ cell proliferation. The $CD8^+$ will differentiate following PGE_2 production from monocytes and γ -IFN from $CD4^+$ cells [82]. In animal models, IFN- β may be a T_S factor necessary for stimulating CON-A induced suppressor cells, that subsequently function via a suppressor immune response substance (SIRS)-like IFN- T_S .

factor [83].

Analysis of the effector arm of Ag-non-specific suppressor T-cell responses is revealing equally complex networks of cellular and hormonal interactions (Ag- and Ag-non-specific soluble factors). For example, CON-A induced suppression of PWM stimulated IgG synthesis in mice seems to involve production of an -SH containing SIRS molecule (44 - 55 kda) which is oxidised by macrophages to a disulphide containing active SIRS, which in turn inhibits B-cell IgG synthesis [84]. In man, CON-A activated mononuclear cells produce soluble immune suppressor substances that function via glycoprotein receptors and inhibit T-cell proliferation, called "SISS-T" [85] and inhibit B-cell synthetic responses, called "SISS-B" [86]. It has been suggested that the CON-A induced suppression may even be mediated by γ -IFN in man [87].

Using this assay to compare CON-A induced suppressor activity in various populations, RA NSAID were found to be significantly defective in suppression of IgG and IgM synthesis, in agreement with numerous papers [88-91]. The defective suppression of IgM synthesis was far worse than that of IgG. This is perhaps surprising in view of the fact that RA is characterised by ^{worse} uncontrolled production of ^{than IgM RF} IgG RF [92]. No correlation was found between CON-A induced suppressor activity and disease duration, although various reports suggest that CD8⁺ cells [89,91] and CD45R⁺ cells [90] are low in early RA, which could be an explanation for the defect observed by some [89] at this stage in the disease. The fact that in this project the defect was

found to persist even late into the disease course, when various reports suggest $CD8^+$ cell numbers are not so abnormal [91], would suggest that the functional defect is dependent on other factors apart from $CD8^+$ cell numbers. It is possible that dose/response curves to CON-A for optimal CON-A induced suppressor activity differ in the patient groups compared to the normals. As such it has been suggested that 3 - 4 concentrations of CON-A should be compared, since an apparent lack of response to CON-A could simply reflect a shift in the concentration of CON-A needed for maximum suppression activity; however because of the large quantity of blood required for this assay only one dose of CON-A was used in comparative experiments between patient groups. However, in three RA NSAID patients, in whom enough blood was obtainable, no alteration in dose/response curve was observed. It is also of course possible that, on the day of investigation, the RA NSAID cells were insensitive to CON-A, due to cyclical changes in response to CON-A. As such, reproducibility studies in patients should have been carried out. However, the fact that the majority of RA NSAID were very defective suggests that a genuine defect exists, and that this is not due to cyclical variations. Patients who did not respond to PWM were avoided in this project, thus this could not account for the defective suppressor activity. Equally, one cannot rule out the possibility that the defects are false positive results due to excess spontaneous suppressor activity [53,62,70], particularly since excess spontaneous suppressor cells have been noted in RA [107]. It must of course not be forgotten that a normal or near-normal suppressor activity in vitro does not rule out the possibility of a defect in vivo and it is not known whether

defective suppressor activity reflects disease activity or specific disease aetiology. Longitudinal studies are therefore needed to correlate phenotypic and functional defects.

There was no evidence of more defective CON-A induced suppressor activity in RA NSAID patients with EAD (which usually is the result of vasculitic lesions). Other reports did show more defects in patients with EAD [93]. Considering that increased vasculitic lesions in SLE and systemic sclerosis have been associated with high background ROS production and high factor VIII levels [94], the finding of no differences between +EAD and -EAD may indicate that background ROS production is no greater in RA NSAID with EAD, than in those patients without EAD.

CON-A induced suppressor activity did not correlate with RF levels, thus all patients were grouped together. However some reports suggest that the defect is worse in seropositive disease [93]. CON-A induced suppression of IgG or IgM synthesis did not correlate with any clinical indices of disease activity, apart from suppression of IgM synthesis and ESR, however this does not necessarily imply that they are pathognomically related.

Despite the unanimous agreement in the literature that aging is associated with alterations in the immune system, there are differences in opinion as to what these alterations involve. Of relevance to this thesis is the observation by some authors of decreased numbers of $CD8^+$ cells with age, resulting in reduced suppressor cell function, whilst others suggest that deficient

suppressor cell function [95] as a consequence of age [96] is due to an intrinsic functional defect, despite normal numbers of $CD8^+$ cells [97]. Most agree that aging results in an increased production of auto-Ag and is associated with increased B-cell reactivity, leading to auto-Ab production and immune complex formation. Thus aging is frequently associated with an increased incidence of autoimmune disease. As such, a relationship between age and defective suppressor activity in RA NSAID was sought: however, none was found to exist.

Some caution needs to be given in interpreting results from RA NSAID patients - and even perhaps those from AS/PSA patients, many of whom were also on NSAIDs as and when needed -. There are in fact conflicting reports on the influence of PGE_2 on suppressor activity: some reports suggest that PGE_2 is a necessary differentiation factor for the induction of certain suppressor cells, e.g. PWM-induced T_S for inhibiting T-cell proliferation [82], and for stimulation of spontaneously active T- and non-T suppressor cells that regulate IL-2 production [64,65]. Others suggest that it reduces the generation of CON-A induced suppressor cell activity in proliferative indicator systems [98,99,100], since in vitro indomethacin treatment of cells increased CON-A induced suppressor cell activity [101], and in vivo treatment of an RA patient with indomethacin improved subsequent CON-A induced suppressor activity by stimulating the proliferative response of untreated cells and increasing the inhibitory effects of the CON-A treated cells [108]. Still others would suggest that exogenous PGE_2 might inhibit PGE_2 -producing radiosensitive T_S cells that regulate IgM RF synthesis, and that NSAID treatment could stimulate this [102].

PGE_2 might inhibit PGE_2 -producing glass adherent suppressor cells (T, B, or monocytes) that inhibit CD4^+ cells γ -IFN production, and PGE_2 might inhibit T_S function controlling mitogen stimulated T-cell proliferation [103] and EBV-stimulated T-cell proliferation [104]. NSAID treatment might inhibit these adherent suppressor cells and therefore subsequently stimulate T_S activity. PGE_2 inhibits IL-2 production, but not CD25 expression, if IL-2 is a necessary growth factor for suppressor cell development, PGE_2 could inhibit subsequent suppressor cell induction and NSAID treatment could stimulate it [105]. Enhanced sensitivity of radiosensitive CD8^+ suppressor cells to the stimulatory effects of PGE_2 -producing monocytes [106] could result in excessive suppressor activity and defective IL-2 production, and defective proliferative responses and AMLR that are observed in RA [107]. Some reports have suggested that short lived suppressor activity and PGE_2 -producing monocytes may be the same or overlapping phenomena, but others disagree. CON-A induced suppressor cells are said not to act via PGE_2 , but are said to be regulated by PGE_2 -producing suppressor cells, therefore exogenous PGE_2 could inhibit the latter and the former, whereas NSAID treatment could stimulate the former and inhibit the latter [98]. It may also suggest that defective CON-A induced suppressor activity in RA could be due to excess sensitivity to inhibitory effects of PGE_2 or excess number or potency of PGE_2 -producing cells.

Perhaps the discrepancies are not surprising in view of the bimodal action of the PGE_2 , which acts both as a pro- and anti-inflammatory agent. Thus, if PGE_2 is needed for suppressor cell

induction, NSAID treatment could artifactually worsen CON-A induced suppressor activity, whereas if PGE_2 inhibits suppressor activity, NSAIDs could artifactually increase CON-A induced suppressor activity. However, in this project, suppressor defects were still observed in RA patients who had been on NSAID for greater than 6 months, and no defects were observed in AS/PSA patients taking NSAID. This finding would suggest that NSAID probably have not artifactually decreased suppressor activity, since defects could be observed in AS too, however it does not rule out the possibility that these drugs could have increased suppressor activity, normalising it in AS/PSA patients - who showed mild defects in the first place - and improving it in RA - who showed major defects in the first place. If such is the case, the suppressor cell defect may have been even worse in the RA NSAID group before treatment. Of interest is the fact that one paper showed increased CON-A induced suppressor activity for suppression of total Ig synthesis in AS patients on NSAID compared to normals [28], suggesting that NSAID treatment could be causing stimulation of suppressor activity; however, the same patients showed normal suppression of PHA stimulated T-cell responses [28]. Because of the known heterogeneity of CD8^+ suppressor cells involved in T-cell and humoral cell responses, results of the latter report might indicate that CD8^+ cells suppressing humoral responses are PGE_2 sensitive, but those suppressing cell mediated responses are not, which parallels the known steroid sensitivity of these two populations [1]. However, this interpretation is unlikely in view of the fact that some of the papers looking at the PGE_2 effects on suppressor function, found that it influenced CON-A induced suppression of mitogen stimulated T-cell

responses too [98,99,100]. In the above paper [28] where suppression of the humoral responses was also studied, no experiments were conducted to look at suppression of individual subclasses of Ig. Of interest in this respect is the observation in this thesis that in one RA NSAID patient in whom suppression of T- and B- responses was examined together with the short-lived suppressor assay, defective suppression of IgM synthesis, defective SLISA and defective suppression of T-cell responses - but relatively normal IgG suppression - were found. Thus, it is interesting to speculate that perhaps NSAID treatment improves defective suppression of IgG synthesis but has no effect on suppression of IgM synthesis. In this respect it should be noted that the RA NSAID patients showed more defective suppression of IgM than IgG, and that there was a lack of correlation between the two. This implies that different mechanisms are involved in IgG and IgM suppression, perhaps acting via different subsets of T-cells that show differential PGE_2 sensitivity and regulate different subsets of B-cells, and only those regulating IgG synthesis being PGE_2 sensitive. Alternatively, one could speculate that different subsets of B-cells make specific Ig, and those producing IgM are less sensitive to suppressor stimuli than are IgG producing subsets.

Thus several possible artifacts might be introduced into in vitro suppressor models by NSAID treatment alone. One is an increased suppression in the CON-A induced suppressor assays [101,108,109]. Secondly, there could be a reduced spontaneous suppression of PWM stimulated RF PFC [110]. NSAID could decrease PWM-induced suppressor activity for inhibiting CD4^+ cell proliferation [82], and perhaps

inhibit mytomycin-C, radiation-sensitive spontaneous suppressor cells that regulate IL-2 production [107]. Because good correlation in time-course of exposure to CON-A was found between proliferation in response to CON-A and generation of suppression in response to CON-A [101], it has been suggested that one should avoid patients showing hyporesponsiveness to CON-A in proliferative assays when looking at CON-A induced suppressor activity, since these patients would probably show poor suppressor activity not because of defective T_S cells, but due to excess PGE_2 (known to inhibit T cell proliferation) or excess sensitivity to inhibitory effects to PGE_2 . It would also suggest that patients on NSAID should be avoided since by stimulating subsequent mitogen-stimulated proliferative responses, it could falsely improve suppressor activity [101].

Different effects may occur when NSAID are given in combination with second line drugs, as often occurred in patients in this project. This could result in improved spontaneous suppressor activity, and increased HAGG stimulated B_S activity of PWM stimulated RF PFC [110].

Therefore, caution needs to be given when analysing data from in vitro suppressor assays using cells from patients on NSAID treatment. What one really would need to investigate are patients with active synovitis on no treatment at all, they of course are rarely available since they would only be on no treatment if by personal preference. A second possibility was felt ethically incorrect, that is to withhold treatment for the purpose of research. Interestingly, in one paper where NSAIDs were withheld for 36 hours and none of the patients were on second line treatment for at least three months or steroids for at

least one month, CON-A induced suppressor activity for suppression of CON-A stimulated autologous T-cells proliferation and for suppression of MLR, and SLISA were still shown to be defective [55]. As such, RA NSAID remain the population used in studies investigating immunoregulation in RA.

Further caution needs to be given regarding the use of PWM in the indicator system. PWM is a polyclonal B cell stimulator that induces the differentiation of B cells in various maturational stages, and many of these cells are mature lymphocytes which are committed to high rates of Ig production [111], which might each require additional or different regulatory signals. Furthermore, only a small percentage (50%) of the total B-cell population is stimulated by PWM, and the possibility exists that Ig production by other classes of B-cells (not stimulated by PWM) may vary in their kinetics and/or susceptibility to CD8⁺ mediated suppression that is not tested by this assay. Thus, the PWM stimulated indicator system represents probably a very complicated model for studying regulatory effects, and may not give complete information. Thus, perhaps, other B-cell activators should also be used to more fully assess regulatory mechanisms in RA, particularly in view of the fact that excess CD5⁺ B-cells are observed in RA [23], which are an immature subpopulation of the B-cells and may be modulated differently to PWM⁺ B-cells examined by this assay. Assays therefore need to be devised to look at immunoregulation of the CD5⁺ cells, particularly in the knowledge that these cells are committed to auto-Ab production [25] (e.g. RF) known to be raised in RA, and that these cells may well be important in the setting-up of the immune

response repertoire by a framework of self-reactivities and of reactions with external Ag via an interconnecting idiotypic network. Thus, defective suppression of these cells - whether by T_S cells or Fc mediated suppression - could allow their uncontrolled differentiation into RF producing plasma-cells.

In agreement with the literature [28], AS/PSA patients showed no suppressor defects of IgG or IgM synthesis. Likewise, Victorino [112] - studying SLISA and CON-A induced suppression of T-cell responses in patients with infectious diseases without immunological disorders (pneumonia, chronic untreated amoebic colitis and urinary infections) - showed no alterations in suppressor cell functions. His report, together with results in this project, suggest that inflammation per se does not lead to suppressor cell dysfunction.

A control SLE group showed very significant defective suppression of IgG and IgM synthesis in agreement with several reports [12,113]; in agreement with a recent report [12] these patients showed more defective suppression of IgM than IgG synthesis.

The RA D-PEN/AU patients showed defective IgG suppression but normal IgM suppression. It was proposed that increased drug doses or alternative drug treatment should be considered. In the two patients showing worst activity, one had been on D-PEN for only 4 months - which could indicate an insufficient length of time for a beneficial effect to be seen -, and the other probably required altered treatment since she had been on gold salts for 37 months. Interestingly, both patients were seronegative (a condition associated with milder RA),

and synovitis was mild in one and absent in the other, suggesting surprisingly that immunological defects can persist despite clinical improvement in terms of synovitis. This is possible in view of the long half life of T-cells, thus functionally defective T-cells could persist after reduced inflammation and reduced ROS production. The fact that IgM - but not IgG - suppression was defective in this patient group could imply that sulphydrate drugs preferentially normalize T_S activity involved in regulating IgM synthesis: this would seem strange if a common mechanism is proposed for the mode of action of sulphydrate drugs, namely the reduction of oxidised functionally important -SH groups on CON-A induced suppressor cells.

Defective CON-A induced suppression of mitogen stimulated T-cell responses was also observed in the few RA patients who were studied, in agreement with various reports [88].

It is hypothesised that the CON-A induced defective suppressor activity of B-cell responses in RA NSAID and some RA D-PEN/AU is due to oxidation of functionally important membrane -SH groups on T_S cells, particularly in view of the ameliorating effect noted when 2-ME or D-PEN were added to cultures of RA NSAID cells, or when 2-ME was added to those cultures from RA patients on sulphydrate drugs still showing defective suppressor activity. This possibility is further substantiated by the fact that RA patients on sulphydrate drugs showed less defects than those on NSAID treatment, and that PHMPSA treatment of normal cells blocked the activity of the suppressor cells, and finally by the fact that serum -SH levels correlated with CON-A induced suppressor activity when all patients and normals were

considered together. It is likely that -SH group oxidation of CON-A induced T_S cells controlling mitogen stimulated T-cell responses is also responsible for the defects shown here, since 2-ME treatment in the few RA NSAID patients studied did ameliorate this defect.

Interestingly, in the one RA NSAID patient where all suppressor assays were used, defective SLISA, defective CON-A induced suppression of IgM and mitogen stimulated T-cell proliferation but relatively normal IgG suppression were observed. Where the defects in this patient were more prominent, 2-ME treatment had the greatest ameliorating effects. This patient is interesting for two further reasons. Firstly, she had secondary Sjögren's syndrome - a condition defined as "dryness of eyes and mouth as a result of lymphocyte proliferation and infiltration into the exocrine glands where increased IL-2 levels and CD25 expression is observed", commonly associated with reduced $CD8^+$ cell numbers and defective suppressor T-cell function [114], also defective AMLR and increased $CD5^+$ cell numbers, leading to B-cell hyperactivity reflected in polyclonal hypergammaglobulinemia, particularly IgG1, and auto-Ab production, including IgM RF specific for IgG1 responsible for the vasculitis and neuropathy that often occur. Secondly, she served to illustrate the need to look at several suppressor assays in the same individual in order to better assess the suppressor cell status, because if only suppression of IgG had been reported, the investigator would have thought that the suppressor cell status of this patient was not particularly defective. However, by reporting results from other assays, defects were noticed. The lack of particularly defective IgG

suppression is in agreement with one report [115]. Unfortunately, it was not possible to perform such a complete analysis for all subjects due to insufficient blood. Likewise, in another report [116] defective CON-A induced suppression of T-cell proliferation in Crohn's disease was observed, but in the same disease no defect in suppression of IgM synthesis was observed [116]. This further illustrates that suppressor systems influencing T- and B-cells are probably distinct [66,117] and that the presence of an impairment of a given suppressor cell function does not preclude the existence of other cells exerting suppressor functions [118-120].

Defective SLISA was also observed in RA NSAID patients in agreement with various reports [54], and may be of more biological significance than CON-A induced suppressor activity, since the former is thought to measure spontaneous suppressor cells. However, the CON-A induced suppressor assay may well be of biological relevance in that it represents latent immunological suppressor activity, and the suppressor cells for the suppression of PFC [121] and T-cell proliferation [122,123] appear to be drawn from the same population of T-lymphocytes activated in the AMLR. The AMLR is a physiologically important reaction producing helper, suppressor, and cytotoxic lymphocytes when T-cells proliferate in response to xenoantigens on non-T cells, and is important for maintaining tolerance. As such, the CON-A induced suppressor cell assay may indirectly measure suppressor activity in the AMLR. One could again speculate that defective SLISA in RA NSAID indicated a common mechanism for defective activity, namely oxidation of functionally important -SH groups, but 2-ME was

not added to such cultures to confirm this; however, indirect evidence for this comes from the fact that RA patients on sulphydrate drugs showed normal SLISA. A control SLE group also showed defective SLISA, in agreement with other reports [124].

Because of the possibility that the hyporesponsiveness of SLE and RA NSAID to mitogenic stimulation per se could possibly account for the defective SLISA observed, correlations between proliferative responses to CON-A and SI were sought. In common with Victorino [112] the conclusion was that in normals SLISA was evident in those individuals who exhibited both high and low responses to CON-A, and that even at low proliferative responses equivalent to those obtained with RA or SLE cells, normal SI were observed. Thus showing that reduced ^3H -TdR incorporation per se does not lead to low SI, suggesting that the assay is not an artifact. Furthermore, the inverse relationship found within normals, between the suppressor activity and the mitogen response, is the expected one, were short-lived suppressor cells to be affecting the response to mitogens in vitro. Two other research groups [125,126] have reported a similar inverse relationship in normals. However, this relationship does not appear to hold for RA and SLE: in such diseases factors other than short-lived suppressor activity must be responsible for the depressed lymphocyte responses to the mitogens, like changes in the capabilities of effector cells, and in vivo prior activation or the presence of soluble substances produced by macrophages that inhibit lymphocyte reactivity in vitro, as recently shown in SLE [127].

A review of the literature shows that sulphydrate drugs improve

and in some cases enhance suppressor activity of RA patients compared to normals in various in vitro models. These drugs improve SLISA for suppression of T-cell responses [129], improve spontaneous suppressor activity for suppression of PWM stimulated RF PFC [110], improve CON-A induced T-cell suppression of ^{both} T-cell responses e.g. PHA stimulated proliferation [128,129] & MLR [68] and B-cell responses - that is Ig secretion - [11], but decrease HAGG stimulated B-cell suppression of PWM stimulated RF PFC [110]. Longitudinal studies need to be carried out to see whether sulphydrate treatment causes improvement in suppressor activity as assessed by various suppressor assays, and whether this amelioration parallels a normalisation of membrane and serum -SH groups. Sulphydrate drugs preferentially up-regulate fast reacting -SH groups [130]. Whether these are important in cellular functions, or whether it is the slow reacting -SH groups that are more important, remains to be seen. It could be speculated that if slow reacting -SH groups are more important for cell function, then this is the reason why these drugs do not cure the disease.

Further evidence for a common mechanism for defective suppressor activity in RA came from the fact that a trend towards a correlation between the CON-A induced suppressor activity for suppression of B-cell responses and SLISA was observed in this project, and from a previous significant correlation observed by another author [112] between CON-A induced suppression of T-cell proliferative responses and SLISA in 12 patients with inflammatory bowel disease, which suggests that the two functions may be related. The relationship between these two tests has not received much attention in the

literature, and although defects in both, within an individual disease have been observed (e.g. SLE and chronic active hepatitis), the tests were rarely performed in the same patient, thus the actual relationship could not be assessed [124,126,131-133]. In other studies, in contrast, excess SLISA has been detected - for example in patients with disseminated fungal infections [134], in whom a defective CON-A induced suppressor activity has been said to be found [118]. Other workers who looked directly at the relationship between SLISA and CON-A induced suppression of T-cell responses [72,124,135] found that pre-incubating cells for 24 hours before exposure to CON-A reduced their capacity to be induced by CON-A to act suppressively, suggesting that the CON-A inducible suppressor cells may themselves be short-lived! However, after 24 hours in culture the situation alters, and CON-A induced suppressor cells are easily activated. Even after an 8-day pre-culture period these cells can be activated [1], suggesting that a considerable number of spontaneous suppressor cells die in the first 24 hours in culture, perhaps releasing transiently reacting suppressor substances into the culture medium that in turn prevent activation by CON-A of other suppressor cells. After this, the putative suppressor cells die more slowly, so that CON-A induced suppressor activity can occur.

To prove that defective CON-A induced suppressor activity was due to surface -SH oxidation, the effects of both PHMPSA and H_2O_2 were assessed. Despite their different modes of action, both were found to inhibit CON-A induced suppressor activity of B-cell responses in a dose-dependent manner in both normals, AS and RA D-PEN/AU patients.

The fact that H_2O_2 often did not totally abolish suppressor activity is probably due to the fact that CON-A induces mainly $CD8^+$ cells but perhaps also $CD4^+$ to function suppressively, and both these cells may coexist with other non-T suppressor cells, like neutrophils, monocytes, macrophages and B-cells, all of which are less sensitive to inhibition by H_2O_2 than $CD8^+$ cells [136]. Leaving H_2O_2 in RA NSAID cultures caused a loss of suppressor activity at lower H_2O_2 doses ($1 \mu M$) than for the other groups ($10 \mu M$), suggesting that many -SH groups may have already been oxidised. The effect of organic mercurial reagents on CON-A induced suppressor activity has not been investigated before, however the effects of H_2O_2 have, and the results agree with those found in this thesis [137]. In view of the fact that physiological concentrations of H_2O_2 - particularly in SF - may be greater than $612 \mu M$ [138], the effects shown here using H_2O_2 in the range $10 \text{ nM} - 100 \mu M$ are likely to be physiologically relevant. One contradictory piece of evidence comes from Goust [139], who showed that oxidising agents can stimulate suppressor activity and T-cell proliferation.

It is interesting to note that the normals studied showed different degrees of sensitivity to PHMPA: in some there was complete loss of CON-A induced suppressor activity, in others very little loss, and in some even stimulation. Experiments were also carried out to assess whether -SH groups were important in the induction of suppressor cells in normals and AS. Again, the results were variable, but in the majority of subjects no effect was observed. It was observed that a lack of -SH sensitivity in CON-A induced suppressor

activity does not necessarily correlate with a lack of sensitivity in CON-A induced suppressor cell induction. Despite the fact that, in the H_2O_2 experiments, H_2O_2 was added throughout the pre-culture and second culture, it is likely that the effects observed were akin to those obtained with PHMPA, that is H_2O_2 affected CON-A induced suppressor activity but not induction. To prove this, experiments could have been carried out adding H_2O_2 to the second and not the first - or the first and not the second - culture respectively. However, this was not done since continuous presence of H_2O_2 was thought of more physiological relevance. It could be argued that H_2O_2 was affecting medium components, particularly in view of the fact that mercaptoalbumin seems essential for lymphocyte growth (Hewlett's hypothesis, personal communication).

The fact that PHMPA inhibited CON-A induced suppressor activity but not induction would imply that in RA CON-A induced T_S cells are successfully produced but become non-functional due to -SH oxidation. To explain excess radiation-sensitive IL-2 production regulating spontaneous suppressor cells in RA [107], one would have to suggest that these cells do not possess surface -SH groups relevant for their function, or that -SH group oxidation stimulates rather than inhibits them.

The lack of an effect of PHMPA on CON-A induced suppressor cell induction, but the significant effect on the activity of such cells for suppression of B-cell responses, is surprising in view of several facts. Firstly, the known inhibitory effects of PHMPA on certain monocyte functions [8,177] could imply that monocytes are not needed

for suppressor cell induction - whereas some authors report that monocytes are in fact needed. Secondly, there is well documented evidence that mitomycin-C treatment of CON-A induced suppressor cells does not affect their inhibitory activity on B-cells responses [70,140], whereas mitomycin-C before induction blocks their subsequent ability to suppress B-cell responses [141]. This suggests that proliferating cells are needed for induction but not the activity of suppressor activity for B-cell responses. In other words, the data using PHMPSA in this project is directly opposed to the data using mitomycin-C. This at first may appear alarming, but one must remember that mitomycin-C inhibits cell proliferation by inhibiting DNA synthesis, a mechanism far removed from that operating when PHMPSA is used, thus results obtained using these two substances cannot be compared. In view of the controversy that continues to surround this area, since a recent report [142] suggests that the activity of CON-A induced suppressor cells for suppression of B-cell responses is inhibited by mitomycin-C treatment - which would be akin to results in this project, - it was decided to investigate the effects of PHMPSA treatment on lymphocyte proliferation.

PHMPSA, whether left in cultures or following pre-treatment of cells, was found to inhibit proliferation in response to PWM, CON-A, anti-CD3 Mab and PHA in a dose-dependent manner in normals, agreeing with previous reports [8,9]. That this effect may be associated with oxidation of functionally important surface -SH groups is likely, in view of the similar dose-dependent inhibition observed either when H_2O_2 was left in cultures or when cells were pre-treated with it.

Further evidence for this hypothesis comes from the fact that defective mitogen stimulated proliferation in RA NSAID was normalised by 2-ME treatment, in agreement with previous reports [8]. This latter phenomenon is odd in view of the lack of amelioration of inhibition of proliferation by 2-ME following D-PEN plus Cu^{++} treatment [9], since this combination is known to inhibit T-cell proliferation by production of H_2O_2 [143], which is therefore akin to one of the mechanisms thought to be responsible for defective proliferation in RA NSAID, namely due to high background ROS production from activated neutrophils and monocytes. This hyporesponsiveness to mitogens in vitro in RA NSAID patients is a well known phenomenon [144-147] and the main reason has been proposed to be due to prior activation of T-cells in vivo. This seems likely in view of the significantly higher background ^3H -TdR incorporation observed in RA NSAID patients in this project compared to controls (data not shown), and also found by others who also observed this in SF lymphocytes [20,21,22]. Further evidence for prior activation of lymphocytes comes from the fact that PBMNC from RA NSAID patients were shown to possess high levels of the activation antigens HLA-DR and CD25, in common with others who also showed raised transferrin receptor (CD71) levels [147]. Enhanced expression of these activation antigens has been particularly shown in the joints [148,149,150] perhaps reflecting the known raised γ -IFN and IL-1 levels there [151,152]. However, the raised activation antigen levels in the circulation [26,27,149,150] is difficult to explain in terms of raised serum γ -IFN and IL-1 levels, since the literature is conflicting in this respect. Immunological stimuli cause T-cells to express HLA-DR antigens, and since the expression of such antigens

seems to be raised in RA, there could be in vivo activation by a disease inciting agent or alternatively T-cells are activated by inflammatory products of the disease process. In this case increased DR expression is merely an epiphenomenon. HLA-DR expression may or may not have a pathogenic role in the disease process but could merely reflect a continued state of immune response to environmental or self-antigens which in turn lead to immunological abnormalities and predispose to autoimmunity. Raised soluble CD25 Ag have also been found in RA circulation and SF from activated T- and B-cells and monocytes, reflecting an ongoing immune response [153,154]. It has been postulated that soluble CD25 provides a negative feed-back to the proliferative response, either by binding IL-2 [155] or removing receptors from the cell surface [156], soluble CD25 may be associated with the defective mitogen induced in vitro proliferative responses that occur in RA. Raised soluble CD25 has been said to correlate with disease activity, but that ESR is a better indicator, since soluble CD25 remains high even when clinical improvement occurs [157]. A 27 kd soluble form of CD8 is also found in the serum and SF of RA, it reflects the presence of activated CD8⁺ cells [158] and correlates with disease activity. However, since this form of CD8 raises again during remission, it is likely that different populations of T-cells release it during active and remission phases [158]. The presence of soluble CD8 is indicative of effector/target cell interactions and low levels are found even in normal serum [159]. Soluble CD8 may reflect cytotoxic cell activity important in the clearance of a primary viral infection, or may reflect suppressor T-cell activity [160]. Finally, the presence of IL-2, B-cell growth factor, B-cell differentiation and

γ -IFN in the joint fluid, all reflect in vivo T-cell activation.

However, in contradiction with the hypothesis that -SH groups are involved in proliferation, DTNB and GSSG, known to react with -SH groups, have been found not to inhibit proliferation or Ig production, only at very high concentrations [9].

The use of a variety of mitogens to investigate peripheral blood proliferative responses, was due to their different modes of actions and different target cell preferences. The relative reactivity of PHA to CON-A is said to be 1.5:1 at optimal doses, and this was confirmed in this project. At optimal concentrations, they show different preferential stimulation: PHA preferentially activates T_H cells - particularly $T_{S/I}$ -, CON-A preferentially stimulates T_S and T_C - and of the $CD4^+$ population more $T_{S/I}$ - and is more monocyte dependent than PHA. PHA and CON-A are therefore called T-cell mitogens. PWM stimulates T_H and B-cells equally, and is thus called a T-cell dependent B-cell mitogen. At suboptimal concentrations these three mitogens preferentially stimulate different cell types: PHA stimulates mainly T_γ cells (usually T_S), CON-A stimulates T_H and PWM stimulates T_S and B-cells. All three mitogens are thought to mimic events in normal cell activation via the stimulation of CD3 and/or CD2 receptor (PHA-P used in this project at optimal concentration stimulates via CD2 and, at supraoptimal concentrations, via CD3 as well; PHA-M only stimulates via CD3 receptors). All these are said to activate 40 - 60% of T-cells. A recently developed Mab to CD3 appears of more physiological relevance, since it stimulates T-cells directly via binding to the δ -chain of the T-cell receptor (thus it is more analogous to antigen

stimulation than the other mitogens in use), causing clonal proliferation via an IL-2 dependent autocrine path [161]. Anti-CD3 Mab recognizes 95% of peripheral blood T-lymphocytes or T-cell subsets and is mitogenic in a dose-dependent manner in the range 0.1 - 1000 ng/ml [162]. As such, it was strange that less ^3H -TdR incorporation was found in normals using anti-CD3 Mab compared to PHA. Low concentrations (25 pg/ml) are said to cause CD25 expression but no IL-2 production or proliferation, probably due to lack of cross-linking, since this concentration would result in the attachment of only 10 molecules per cell. At higher concentrations the Fc portions of the Mab are thought to bind to specific Fc receptors on accessory cell membranes, causing stimulation of IL-1 production and - perhaps - PGE_2 . The accessory cells enable anti-CD3 Mab to cross-link CD3/Ti on T-cells thus causing T-cell activation and subsequently regulating CD2 expression [163]. It appears that accessory cells have a second role too, that of stimulating CD2 receptors on T-cells via their LFA-3 Ag (CD58) [164]. This acts as a secondary signal for IL-2 production and induction of DNA synthesis and proliferation. CD2 is said to act as a negative activation signal when stimulated along with CD3 [165], or an alternative antigen and monocyte independent activation signal if stimulated alone [166-169]. The effect of anti-CD3 Mab on different T-cell subsets varies; it stimulates T_C proliferation but inhibits their function; it stimulates T_S and $T_{S/I}$ function but inhibits their proliferation; it stimulates both function and proliferation of T_H cells. This suggests perhaps the existence of either different T-cell receptors on different T-cell subsets and/or of different signal transduction mechanisms. At low and

optimal doses anti-CD3 Mab preferentially stimulates $T_{H/I}$ and $CD8^+$ cells; at higher concentrations it preferentially stimulates $T_{S/I}$ and $CD8^+$ cells. The mouse IgG2a anti-CD3 Mab used in this project reacts preferentially with FcRI receptors on monocytes and is the most commonly used class of Mab for mitogenic studies, since it stimulates in approximately 100% of subjects, whereas IgG1 Mab or IgG2b Mab stimulate in fewer cases [170]. This differential responsiveness is thought to reflect genetic variability in monocyte expression of Fc receptors. Since all RA NSAID responded to IgG2a anti-CD3 Mab to different degrees, some reacting normally and some showing hyporesponsiveness, the FcRI expression cannot be particularly abnormal in RA in agreement with the literature showing raised Fc expression [180], nor its ability to bind ligands, such as this Mab. This of course tells us nothing about FcRI function. In general, Fc receptor function is said to be defective in RA, as shown by defective Ab-mediated feed-back suppression [10], which may be due to decreased -SH groups known to be important in this receptor function [171].

PHMPA inhibited all mitogenic responses in normals equally to 80% at 50 μM , and totally inhibited proliferation at 100 μM . H_2O_2 caused inhibition of proliferative responses at 10 μM , but the cells were only 50% viable, and caused total inhibition at 50 μM - but the cells were all dead. That the inhibition was due to blockade or oxidation of surface -SH groups respectively is substantiated by reports by Noelle et al. [172,173] using mice, showing high oxygen tension or O-phenanthroline-cupric complex oxidises surface -SH groups and inhibits CON-A induced proliferation, which is substantially

reversed by 2-ME or glutathione. Mitogenic effects of H_2O_2 were observed at low doses ($0.1 - 1 \mu M$), which could be due to the known stimulatory effects of low dose H_2O_2 on IL-2 production [32]. H_2O_2 inhibition of proliferation began to occur at lower concentrations of H_2O_2 when using anti-CD3 Mab, starting at $1 \mu M$ as opposed to $10 \mu M$ with the other mitogens; this could be due to the fact that although all the mitogens used are accessory cell dependent - and accessory cell functions are known to be -SH dependent [8] -, the mitogenic action of anti-CD3 Mab may be more accessory cell dependent, thus more susceptible to agents that block accessory cell function. This is reasonable in view of its interaction with Fc receptors on accessory cells which have important -SH groups for their function [171]. Also, anti-CD3 Mab may increase cell surface -SH group accessibility to PHMPA and H_2O_2 interaction by altering the conformational equilibrium or vertical displacement of some functionally important -SH bearing proteins, therefore more suppression is observed following stimulation with this ligand [174].

A number of differences were observed in this project compared to other reports using H_2O_2 . Cytotoxicity was detectable following a 1-hour incubation at $37^\circ C$ or after a 72-hour incubation from $10 \mu M$ H_2O_2 onwards, with total ablation of viability at doses $\geq 50 \mu M$, whereas Staite [136] found that a dose as high as $320 \mu M$ for 1 hour did not appreciably affect viability using a sensitive fluoresceine diacetate method; Zoschke [175] found that doses $< 40 \mu M$ for 1 hour at $37^\circ C$ were sublethal using trypan blue. However, in this project, using ethidium bromide and acridine orange, sublethal doses were in

the range 10 nM to 1 μ M. As such, not surprisingly, differences in susceptibility to proliferation were observed in this project compared to other reports. 5% inhibition was in fact noted at doses as low as 0.01 and 1 μ M H_2O_2 , and 50% inhibition was found at 50 μ M, with a stimulatory effect observed at 0.1 μ M, whereas Zoschke [175] found that a low dose stimulatory effect occurred at 5 μ M, and that 50% inhibition of proliferation occurred at 40 μ M. The results in this project more closely paralleled those found by the aforementioned author using pure lymphocytes, and this author suggests that the greater sensitivity to H_2O_2 of fractionated lymphocytes compared to unfractionated lymphocytes is due to the scavenging capacity of resting monocytes in the latter population. One possibility to account for these differences is that polystyrene tubes were used for the assays, which may have activated monocytes (and cause ROS production), making them less able to scavenge ROS [137] and making local ROS concentrations higher than predicted.

The fact that inhibition of proliferation using PHMPA and H_2O_2 was similar, lead to the speculation that a common mechanism of inactivation of T-cell proliferation operated in all cases, apart from the known inhibitory action of PHMPA on accessory cell function required in each case. The observation that defective proliferation in RA NSAID is normalised by 2-ME treatment in vitro, and that RA patients on sulphydrate drugs show normal proliferative responses, could possibly reflect inhibition of mitogen-stimulated lymphocyte proliferation due to inhibitory products from activated monocytes - e.g. ROS [175a,176] and PGE_2 [101] - and from activated neutrophils that were frequently observed to contaminate ^{RA NSAID} PBMNC preparations.

However, products from such cells cannot wholly explain the hyporesponsiveness, since mixing experiments have shown that hyporesponsiveness is still present even in the presence of non-activated normal monocytes, suggesting inherent defects in lymphocyte proliferation in RANSAID [147]. Several mechanisms could exist whereby

equal inhibition of proliferation by agents reacting with -SH groups could occur following stimulation with different mitogens. One is the inhibition in the expression of constitutively expressed T-cell Ag - like CD3, CD4, CD2, CD8, CD45R, CDW29 - and of inducible activation Ag - like HLA-DR and CD25, important in cell interaction and proliferation -. As such, their expression was assessed following PHMPSA treatment. Since phenotypic markers are increasingly being shown to be more than just adhesion Ag, but have important immunoregulatory functions too (Fig. 5.1), the expression of other surface markers - e.g. CD11b (mainly on monocytes), CD57 picked up by HNK-1 (mainly on NK-cells) and CD37 (on B-cells) - was also studied. Also the possibility exists that PHMPSA and H_2O_2 caused inhibition of T-cell proliferation, by inhibiting the induction of inducible activation Ag - e.g. HLA-DR and CD25 -, or perhaps the induction of the high affinity IL-2 receptor, by inhibiting CD25 and P75 interactions. Another possibility is that these reagents could have inhibited interleukin production needed for HLA-DR and CD25 up-regulation, i.e. γ -IFN and IL-1, IL-2 respectively. Alternatively, inhibition of proliferation could be due to inhibition of binding of interleukins to their appropriate receptors by blocking -SH groups important in the binding sites; or finally by inhibiting signal

FIGURE 5.1

FUNCTIONAL ROLES FOR SOME PHENOTYPIC MARKERS

ANTIGEN	FUNCTIONS
CD3	<ul style="list-style-type: none"> - needed for accessory cell dependent, Ag-specific, MHC restricted, IL-2 dependent T-cell activation - signal transduction mediated via ζ-molecules - regulates CD2 expression on T_H, T_S, T_C, $T_{S/I}$ cells - stimulates PK-C activity causing serine and threonine phosphorylation of CD3, CD4, CD8, CD45 and MHC I antigens
Ti	<ul style="list-style-type: none"> - binds antigen and recognises MHC class II antigens
CD2	<ul style="list-style-type: none"> - negative activation signal when stimulated together with CD3 - positive activation signal when stimulated alone - stimulates PK-C activity causing serine and threonine phosphorylation of CD3, CD4, CD8, CD45 and MHC I antigens
CD28	<ul style="list-style-type: none"> - alternative activation signal pathway in T-cells
HLA-DR	<ul style="list-style-type: none"> - immune regulation by allowing antigen presentation - regulation of CD2 expression
CD4	<ul style="list-style-type: none"> - immune regulation by recognition of MHC class II antigens - associated with $P56^{lck}$, allowing its movement nearer to CD3 where it phosphorylates tyrosine on the ζ-protein of CD3 to regulate its function
CD8	<ul style="list-style-type: none"> - same as CD4, but it recognises MHC class I antigens
CD45RB	<ul style="list-style-type: none"> - may regulate CD3-T_I, CD4, CD8, CD2 via intrinsic phospho-tyrosine phosphatase activity
CD10	<ul style="list-style-type: none"> - cell adhesion marker with intrinsic enzymic properties
CD16 (FCR III)	<ul style="list-style-type: none"> - Adhesion molecules to bind Ab or Ig on B-cells - Mediate endocytosis - Involved in signal transduction
CD23 (FCR II)	
CD64 (FCR I)	

transduction mechanisms from receptors (e.g. interleukin receptor) by inhibiting functionally important -SH groups closely associated with G-transducing proteins. This is a reasonable possibility in view of the fact that -SH groups have been found on P21 of V.ras oncogene which has sequence homology with G-proteins and appears to be in the vicinity of the GTP binding domain [178].

PHMPA was found not to affect the expression of any of the phenotypic markers under consideration, in agreement with unpublished observations by N. Goulding in our laboratory. Although complementary parallel studies looking at the effects of H_2O_2 were not carried out in this thesis, others have done so. Staite et al. [136] found that 320 μM H_2O_2 incubated with lymphocytes for 1 to 2.5 hours at 37 °C (a comparable incubation period to that used in this thesis) did not alter CD4, CD8, CD2, CDW29 binding or expression as assessed using Becton-Dickinson Leu-3ab, Leu-2b, Leu-5b and Coulter 4B4-RD1 Mabs respectively. Nor did H_2O_2 cause phenotypic switching of CD8 to CD4. However SRBC-rosette formation was inhibited by H_2O_2 . Thus probably Leu-5b detects T11/2, not T11/1 which is the Ag for SRBC. It is therefore possible that H_2O_2 could be inhibiting either the binding or expression of T11/1. It is unlikely that H_2O_2 affected the induction and expression or binding capacity of the third cryptic epitope of CD2 called T11/3, recently designated CD2A, since the latter is only expressed on activated cells and this work used fresh cells [168]; also T11/3 is not involved in SRBC reactions. In another paper by Gougerot [179] lymphocytes were incubated with normobaric oxygen for 24 to 72 hours, and this did not affect the expression or binding of

constitutively expressed Ag, e.g. CD8, CD3, CD4 using IOTTECH Mabs. This author also observed that 2-ME treatment had no effect on the expression or binding properties of these antigens. The results using 2-ME on CD8 expression and binding are interesting in view of the fact that CD8 is a known disulphide linked homodimer, thus it might have been expected that 2-ME would release the 2 monomers into their thiol forms. However, since IOT8 binding was unaffected by 2-ME, one must assume that this Mab only needs to bind to an epitope on one monomer and does not require cross-linking across the two. In this respect, it would have been interesting to see the effect of 2-ME on Mab 9.3 binding to the CD28 receptor and Mab binding to the Ti receptor, known disulphide linked heterodimers, and also SOTON-M2 binding, said to detect CD11 antigen, which are the C3bi receptors on monocytic cells and said to contain a disulphide bond. If the Mabs require recognition of epitopes on both monomers, then 2-ME might inhibit binding. It must of course be remembered that, just because antigens can still be detected after PHMPSA or H_2O_2 treatment, does not mean that they are still functional, e.g. excess FcR expression is present in RA, but is poorly functional in Fc mediated feedback suppression [10].

Neither PHMPSA nor H_2O_2 affected the induction or expression of CD25 or HLA-DR Ag on mitogen stimulated cells or "educated" cells. On mitogen activated T-cells anti CD25 Mab will be binding to CD25 present in both low affinity IL-2 receptor binding sites (of which there are 30,000 to 60,000 per cell representing 80 to 90% of total receptor sites, [181]), and high affinity IL-2 binding sites (of which there are 2,000 to 4,000, representing 5 to 10% of total receptor sites

[181]), if one assumes that the amount of Mab bound^{to} the high affinity sites is visibly detectable and not swamped by the binding to excess low affinity sites, then PHMPA and H_2O_2 did not inhibit Mab binding to either of the receptor sites, which would mean that -SH groups are not involved in the binding sites of either receptor. However, because of the insensitivity of the fluorescence technique, a small loss in binding to high affinity sites may not be visible. These results are in direct contrast by those of Gougerot [179] who states that "...incubation with normobaric oxygen for a similar period - i.e. 24 - 72 hours - caused inhibition of inducible activation antigens, namely CD25 and transferrin receptors". Perhaps in keeping with the idea that ROS inhibits CD25 induction are two reports suggesting defective CD25 induction in RA, one report using PHA stimulated PBMNC [182] and the second finding reduced expression of CD25 on SF lymphocytes despite obvious signs of activation in terms of size and HLA-DR expression [148]. These latter authors suggested this was either due to activation beyond the CD25 expression stage, or that expression of HLA DR particularly on CD8 cells was due to mechanisms unrelated to immune stimulation especially in view of normal amounts of γ IFN from stimulated SF T cells; this discordant expression of activation antigens was termed "frustrated activation" [148]. In the light of Gougerot's work [179], the first report could perhaps be interpreted as inhibition of CD25 induction by ROS produced by activated monocytes and contaminating activated neutrophils in the PBMNC preparation - and in the second report due to high local ROS production. However, Gougerot's experiments differed from the ones reported here in a number of ways. He used normobaric oxygen - not H_2O_2 - and more

importantly used Becton-Dickinson anti-CD25 Mab which reacts to different epitopes than DAKOPATTS anti-CD25 Mab in view of the fact that the former anti-CD25 Mab can block IL-2 binding, but not the latter. Gougerot did suggest that the effects he showed may not be direct effects inhibiting receptor induction, but could be secondary to the fact that this normobaric oxygen treatment inhibited T-cell activation. Although neither analysed by this author nor in this project, it is possible that ROS or PHMPSA could affect the mitogen-stimulated induction of the cryptic epitope of the CD2 receptor, i.e. T11/3 which is important in the signal transduction from this receptor, particularly in view of the fact that the CD2 receptor is proposed to play a fundamental role in the regulation of T-cell activation [168,169]. H_2O_2 and PHMPSA could of course affect the induction and/or the expression of other receptors as yet unknown, which may be important in T-cell activation, and thus inhibit proliferation in this way.

The fact PHMPSA and H_2O_2 did not affect HLA-DR and CD25 induction or expression perhaps is indirect evidence to suggest that γ -IFN, IL-1, and IL-2 production are not SH-dependent mechanisms, the latter two being in agreement with two reports from our laboratory showing no effects of 2-ME treatment on human ^{IL-1 and} IL-2 production [8] and no effect of PHMPSA on IL-2 production from mouse cells, and with results in this project. Further evidence for this comes from the finding of a lack of correlation between serum SH and IL-2 production in RA NSAID. However, if thiol status does not affect IL-1 and IL-2 production, it is difficult to understand the conflicting reports that

oxygen and H_2O_2 do affect interleukin production, if a common mechanism is proposed for both effects, namely interaction with -SH groups. In one report it was observed that normobaric O_2 stimulated IL-1 and IL-2 production, but 2-ME had no effect [179]. A second report stated $10 \mu\text{M}$ H_2O_2 stimulated IL-2 production, but 30 to $100 \mu\text{M}$ inhibited it [32].

There are conflicting reports of mitogen-stimulated IL-2 production in RA, some suggesting low, normal or high levels; the differences probably are related to heterogenous populations studied in terms of disease activity, drug treatment, etc. and different methodologies used. In this project no significant defects in IL-2 production in RA NSAID were observed, although RA patients with EAD were slightly more defective in common with a recent report by Kitas [147] showing significant defects in total RA patients and particularly in the EAD subgroup which showed particularly high background CD25 levels and perhaps this caused increased utilisation of the IL-2 produced. It was perhaps surprising that the time course of IL-2 production was found to be similar in RA NSAID and normals and that there was no spontaneous IL-2 production in RA despite T-cell prior activation as evidenced by raised background CD25 and HLA-DR expression, both in this project and in the report by Kitas [147]. IL-2 production in RA NSAID did not correlate with any indices of disease activity or the presence of RF, in common with another report [147]. However, unlike this report, IL-2 was found in this project to positively correlate with age and disease duration, and the latter is not surprising in view of the known greater severity of

immunoregulatory defects in early RA. IL-2 did not correlate with CON-A induced suppressor activity for B cell responses, suggesting no relationship between the two. Longitudinal studies are needed to see whether sulphhydrate treatment causing raised SH levels correlates with improved IL-2 production. However, the finding that PHMPSA did not influence IL-2 production in RA NSAID would suggest that thiol status does not influence the production of this interleukin. IL-2 production in the AS group was similar to normals, in agreement with the literature [183].

The defective proliferation in RA NSAID and defective CON-A induced suppressor activity can therefore not be accounted for as an inhibition of IL-2 production, since no significant defect was observed.

The method adopted to measure IL-2 production in this thesis using "educated" cells has been shown by its originators [184] to be comparable with other assay systems and is specific for IL-2 over a broad range and is not influenced by IL-1. However it is possible that it may detect IL-4 (BSF-1, a T-cell derived lymphokine that acts primarily on B-cells [185]) as well as or instead of IL-2, since IL-4 interacts with lymphoblasts [186]; this is unlikely to be a problem however because the amount of IL-4 required to induce proliferation is extremely high [186], unlike the high sensitivity of "educated" cells to IL-2 levels as low as 0.001 IU/ml, and peak IL-4 production in mitogen-driven systems occurs after 72 hours [187], not at 48 hours, the time of supernatant harvest for maximum IL-2 production, as used in these experiments. The "educated" cells from different individuals

showed considerable variations in $^3\text{HTdR}$ incorporation both in background and IL-2-induced responses. "Educated" cell "reactivity" was however similar in all individuals, i.e. the amount of IL-2 required to induce half maximum proliferation, as noted in [184], and the slight differences between individuals did not matter since a standard curve from the same source was put up on each run.

An alternative explanation for the inhibitory effects of PHMPSA and H_2O_2 on mitogen stimulated proliferation is that they inhibit the ability of receptors to bind their natural ligands, implying that -SH groups are important in the active sites for ligand-receptor interaction. Inhibition of the binding of CON-A or PHA to their receptors is unlikely to account for the inhibition observed in view of two reports showing exposure to normobaric oxygen did not modify their binding [179,188]. A third report showed that diamide did not inhibit ^{125}I -CON-A or ^{125}I -PHA binding [189]. This might indirectly suggest that SH groups are not important in mitogen binding; further evidence for this comes from the fact that pre-treatment of cells with PHMSA did not inhibit CON-A induced suppressor cell induction. One report suggests that CON-A acts via one receptor site to stimulate blastogenesis, and a second to induce suppressor activity. If true, this would suggest that neither site has an -SH group involved in CON-A binding. Still further evidence for the unlikely possibility that -SH groups are involved in receptor binding sites for CON-A and PWM comes from evidence from the structure of both mitogens. CON-A is extracted from the legume "Concanavalia ensiformis" and is a lectin and a mitogen, it is a pure globular protein binding specifically to

glycoproteins or glycolipids on cells containing α -D-glycopyranoside or α -D-mannopyranoside residues. At pH 7.4 it is a tetramer and each subunit has no cysteine residues, thus it has no S-S intramolecular bonds and cannot have free -SH groups. PWM is a mitogen and poor lectin extracted from "*Phytolacca americana* pokeweed" and is a glycoprotein with no free -SH groups. All 66 of its cysteine residues are involved in the production of 33 intramolecular S-S bonds.

IL-1 and γ -IFN binding seem unaffected by H_2O_2 and PHMPSA treatment in view of the fact that CD25 and HLA-DR induction and expression on "educated" cells (Table 4.34) were unaffected and these two interleukins are known to upregulate the expression of HLA-DR [190,191] and CD25 [192,193] respectively. Experiments carried out to see if IL-2 binding to its high affinity receptor was inhibited by PHMPSA revealed interesting results, i.e. there was 32% inhibition of binding to high affinity receptors in HuT cells, suggesting SH groups may be involved in HA - IL-2 interactions. A recent paper suggests that many surface receptors coupled to Gs proteins are S-S linked, and that agonist binding occurs by breaking the S-S bond to form -SH groups with which the agonist then interacts [238]. Since PHMPSA cannot break S-S bonds, the inhibition could be due ^{to} \wedge -SH groups already on the HA receptor. Of interest was the observation that in another system, namely neutrophils, a receptor-driven activation phenomenon via FMLP (to mimic bacterial cell walls, [194]) was inhibited by PHMPSA treatment, but a non receptor-driven event (PMA) was not. The inhibitory effect of PHMPSA on $O_2^{\bullet -}$ from neutrophils is the direct opposite to that shown by Tsan who showed only H_2O_2 production was

affected [195,196], but is consistent with another paper by him, showing that reagents that interfere with -SH groups on serine-proteases necessary for $O_2^{\bullet-}$ production inhibit its subsequent production from neutrophils [197]. The lower $O_2^{\bullet-}$ produced using FMLP compared to PMA was probably due to the fact that $O_2^{\bullet-}$ itself may destroy the biological activity of FMLP by oxidation of a methionine residue in it, thus limiting the extent of $O_2^{\bullet-}$ production by this ligand. The fact PHMPA stimulates $O_2^{\bullet-}$ production after PMA is not understood but has been noted by others with a chemiluminescent technique or using low doses of PHMPA in an H_2O_2 generating system. It may suggest that perhaps following certain stimuli and in the presence of oxidised or blocked certain surface SH groups, ROS production is stimulated, suggesting the process could be autocatalytic. However, PMA, although an analogue of diacylglycerol, does not have an in vivo equivalent and releases specific granule constituents, whereas in vivo usually azurophils are released, so results using it may not be physiologically relevant. Other authors using the same neutrophil system have found other receptor-driven events to be also PHMPA-sensitive, e.g. HAGG-stimulated, to mimic immune complexes (via FC receptors) and opsonised zymosan stimulated, to mimic bacteria or complement (via C3bi receptors) and FMLP stimulated (via chemotactic peptides mimicking N-formyl methionine, the end aminoacid an all prokaryote proteins) ROS production [166,196,198,200]. Greater inhibition following PHMPA treatment was observed in these reports, probably due to methodological differences, since cytochalasin B and Ca^{++} and Mg^{++} were added to the cells in the experiments carried out in this thesis

to increase $O_2^{\bullet -}$ output, but this was not done in the previous reports. Cytochalasin B was added to enable ROS production to occur independently of phagocytosis.

The neutrophil experiments could thus be interpreted in a number of ways, e.g. PHMPSA could be inhibiting receptor-ligand interactions (implying that SH groups are important in the active site of the receptor) and/or signal transduction mechanisms (implying that SH groups on or near G-transducing proteins are blocked). The inhibitory effect of PHMPSA on HAGG stimulation is most likely due to inhibition of Fc receptor function, since -SH groups are known to be important for this. The inhibitory effect of PHMPSA on opsonised zymosan is odd, since -SH groups are not involved in C3bi receptor binding, however an S-S bond is important for its function [199,200].

Alternative explanations for the inhibitory effects of H_2O_2 and PHMPSA on mitogen stimulated proliferation could be due to inhibition of signal transduction mechanisms by inhibiting -SH groups on or near the G-transducing protein linked to the receptor, or inhibition of the interaction of P50 and P75 to form the HA receptor if S-S linked. Experiments were carried out to try to find which of these possibilities apply to the inhibition of cell proliferation. "Educated" cells provided a useful model to investigate these possibilities. Taking "educated" cells which already express the high affinity IL-2 receptor, subsequent proliferation in response to IL-2 was inhibited equally over the range 0-1000 IU/ml following PHMPSA treatment. This inhibition of proliferation could be interpreted as partly due to inhibition of IL-2 binding to its receptor, but since

using HuT cells binding was found to be only 32% inhibited, while proliferation of "educated" cells was inhibited to 90%, one would expect another inhibitory mechanism to be in operation, for example inhibition of signal transduction. The fact that inhibition was equivalent using physiological doses of IL-2 0-100 IU/ml that stimulates the high affinity receptor [201,202] and pharmacological doses [201,202] of IL-2 100-1000 IU/ml (that under certain circumstances are able to stimulate the intermediate affinity receptor to work in isolation [201,202,203,204]) showed that both the high affinity and the intermediate affinity receptors are inhibited from IL-2 binding and/or signal transduction. If the high affinity IL-2 receptor is disulphide-linked dimer of α and β chains, PHMPSA would not be expected to unlock such a bond and release the intermediate affinity receptor to work alone [201,203], thus it cannot be inhibiting proliferation by the breaking of disulphide bonds. The fact that previous reports have shown that even in the presence of anti-TAC high doses of IL-2 can force the intermediate affinity receptor to work alone may suggest that the high affinity receptor is not disulphide-linked, and that the α and β chains can be expressed and function independently of each other, but sometimes function synergistically. Since the "educated" cells are formed solely by FCS stimulation and not by mitogens, one might envisage that not all the intermediate affinity receptors will be engaged to form the high affinity receptors, and some may still be free, so the finding of equal inhibition at high doses of IL-2 suggests that even free intermediate affinity receptors cannot allow proliferation following PHMPSA treatment.

Since inhibition at both physiological and pharmacological doses of IL-2 was also observed when cells were treated with PHMPSA before education to express high affinity receptors, and the fact that the activation antigens CD25 and HLA-DR were not affected, suggests that partial activation of these cells was unaffected by PHMPSA. It is possible that lack of proliferation using such cells resulted from either a partial lack of IL-2 binding to intermediate affinity receptors and correctly formed high affinity receptors and/or inhibition of signal transduction. However, if the high affinity receptor is a disulphide cross-linked dimer formed between functionally important SH groups on P55 (β chain) and P75 (α chain) proteins, one could envisage that PHMPSA treatment although not inhibiting CD25 induction or expression, could inhibit the formation of functional high affinity receptors by blockading SH groups on both proteins. Since intermediate affinity receptors are present even on resting cells [203] and FCS may not be expected to cause all these receptors to be involved in high affinity receptor formation, one might have expected high doses of IL-2 to be able to bind IL-2 and transduce proliferative signals independently of the β chain. Since even at pharmacological doses of IL-2 no proliferation was observed, one must assume that either IL-2 binding to the α chain and/or signal transduction was also inhibited.

One could envisage the possibility that there are intrinsic differences in the properties of P75 in resting and activated T cells and that PHMPSA pre-treatment of cells being "educated" or unfractionated PBMNC, in some way affects P75 activation subsequently

inhibiting its ability to transduce proliferative signals; alternatively P75 activation could involve the induction of yet another as yet not recognised protein, which is itself PHMPSA sensitive, required for IL-2 binding and/or signal transduction.

Turning to the CON-A induced suppressor assay, if proliferating cells are necessary for suppressor action and if the HuT experiments are subsequently confirmed to be true, i.e. PHMPSA inhibits IL-2 binding to the high affinity receptor, and it can be shown that a small inhibition in binding can lead to a dramatic inhibition of cell proliferation, then one could interpret the ability of PHMPSA to inhibit the "activation" of CON-A induced suppressor cells in a dose-dependent manner as merely confirming the view that the assay is an in vitro artifact and that the inhibitory phenomenon observed is merely the result of depletion of IL-2 for use by responder cells by proliferating CD25⁺ T_S cells and it would imply that SH groups are important in the binding site of the high affinity receptor. Evidence for the existence of CD25 on CON-A induced suppressor cells comes from work from Uchiyama [205,205a]. Evidence to suggest that very few IL-2R need to be occupied to mediate proliferation comes from work by Depper who showed that addition of even small amounts of purified IL-2 to antigen-stimulated cultures in the presence of anti-TAC completely abrogated the inhibitory effect of anti-TAC. He was of course not able to measure the amount of IL-2 produced by the cells already at or near the cell surface [204]. If however the HuT experiments are subsequently proved wrong, or a slight inhibition in IL-2 binding cannot lead to a dramatic inhibition of proliferation, it might

suggest that PHMPSA inhibits the activity of CON-A induced suppressor cells by inhibiting signal transduction mechanisms from the IL-2 receptors which may be necessary for their function. Alternatively, IL-2 and IL-2R may have no importance in the activity of suppressor cells and an alternative explanation is required; it is felt that this is more likely since otherwise their induction would also have been PHMPSA-sensitive.

To explain the inability of PHMPSA to inhibit the "induction" of CON-A induced suppressor cells, in the light of results from HuT cells, "educated" cells and proliferative and Ig producing experiments using PBMNC, one would have to hypothesize that proliferation is not a prerequisite for "induction" of suppressor cells and that IL-2 and IL-2 receptors are probably not involved in the early stages of suppressor cell development. If the assay is merely an in vitro artifact, the inability of pretreatment with PHMPSA to inhibit subsequent suppressor cell induction would suggest that it did not inhibit IL-2 binding, since otherwise suppression would have been blocked as in the activity experiment. So, this experiment tends to refute the objections which imply that this assay is an artifact of IL-2 consumption, and tend to suggest that CON-A induction and activation of suppressor cells does not involve proliferating cells. Despite not testing whether added IL-2 inhibited suppressor activity (the direct way to test whether the assay is an artifact), various pieces of evidence suggest that it is not, but represents true suppression by suppressor cells and/or their soluble suppressor factors, including 1) the fact PHMPSA did not always substantially

inhibit suppressor activity in normals; if an artifact, it should have consistently inhibited it; 2) when CON-A induced suppressor cells are harvested at 48 hours, the CD25 antigen will not be maximally expressed; this would only happen at day 2 of the co-culture and IL-2 production from responder cells would only be maximal at day 3 of the co-culture, yet substantial suppressor activity is observed in kinetic experiments even on day 2 of the co-culture in all patient groups and normals, suggesting IL-2 is not necessary for suppression to occur; 3) it would seem more reasonable to expect that total inhibition of binding of IL-2 to its receptor would be needed to explain the total obliteration of activity following PHMPSA treatment were it an artifact, and PHMPSA only partially inhibits IL-2 binding in HuT experiments; 4) the high affinity receptor is only present on proliferating T-cells [205] and there are several reports suggesting proliferation is not needed for suppressor cell activity [70,140], and strong mitogenic responses are not necessary for suppressor cell activity, and good suppression is still observed at non mitogenic doses [1], and suppressor cell activity is preferentially present in high density poorly proliferated cells [208]; 5) there is evidence that functional T_S cells occur even under conditions where proliferation is not occurring suggesting a dissociation between proliferation and differentiation signals for T_S cells [206,207]; 6) a lack of correlation between CON-A induced suppressor activity and IL-2 concentrations (produced by proliferating cells) in RA NSAID and normals (data not shown) found in this project perhaps imply proliferation is not needed for suppression; 7) suppressor cell activity has been observed as early as 6 hours after CON-A treatment

but significantly raised ^3H -TdR incorporation is only detected after 44 hours [101]; 8) since CD25 expression is only up-regulated considerably in proliferating cells, ~~so~~ if proliferating cells producing IL-2 [205,194] (where there is a 8-10-fold increase in CD25 expression and a 20-30% fall in HA expression) are not necessary for suppression expression, it would suggest that the amount of CD25 expression on such cells would be insufficient to reduce IL-2 levels so dramatically that there would be insufficient for responder cells; 9) two papers indicated a direct effect of CON-A induced T_S cells on IL-2 independent cell lines [209,210]. Thus, we suggest that IL-2, CD25 and proliferating cells are not necessary for induction or expression of suppressor cells.

If however IL-2 consumption by binding to CD25^+ in LA and HA receptors on activated T_S cells does account for the induction and activity of suppressor cells, ~~one must assume that~~ ^{But experiments are proved wrong,} activation signal transduction from the P75 protein of the HA IL-2 receptor is not thiol-dependent, but proliferative signals from the P75 protein of the HA IL-2 receptor are thiol-dependent, a lack of effect on the induction could also imply that the HA IL-2R is not disulphide linked; since the same high affinity receptor is involved in both instances, one could envisage its linkage to different G-transducing proteins depending on the state of activation of the cell, and that only transducing proteins used late in the cell cycle are thiol-dependent, e.g. a G_s -transducing protein causing a rise in cyclic AMP in the $G_0 \rightarrow G_{1a}$ transition and perhaps a G_i transducing protein causing a fall in cyclic AMP in the $G_{1a} \rightarrow S$ transition; alternatively the IL-2 receptor

could be linked to the same G-transducing protein throughout the cell cycle, but it could be linked to different second messenger generating enzymes depending on the state of activation of the cell and perhaps depending on which enzyme it is associated with, its conformation could be altered exposing SH groups only important for its function late in the cell cycle $G_{1a} \rightarrow G_s$, e.g. adenylyl cyclase producing cyclic AMP in the $G_o \rightarrow G_{1a}$ transition, and guanylyl cyclase producing cyclic GMP in the $G_{1a} \rightarrow S$ transition to induce proliferation [211]; this is probably the more likely mechanism, since there are many instances where it occurs in biology.

We therefore suggest that one explanation for the effects of PHMPA and H_2O_2 in all the assays used in this project is that partial activation or competence signals (i.e. $G_o \rightarrow G_{1a}$) are not SH-dependent mechanisms, and so IL-2, IL-1 and γ -IFN production, CD25, HLA-DR induction and expression, and "induction" of CON-A induced suppressor cells and activation signals from the IL-2 receptor are not affected by PHMPA or H_2O_2 , whereas some progression signals for proliferation and differentiation (i.e. $G_{1a} \rightarrow S \rightarrow M$) are thiol-dependent mechanisms, e.g. CON-A induced suppressor cell "activity" and proliferative signals from the HA or P75 IL-2 receptor. It is proposed that the different effects of PHMPA on the activity but not induction of CON-A induced suppressor cells implies that IL-2 and IL-2R are not involved, and that other activation antigens expressed later in the cell cycle may be inhibited by PHMPA or H_2O_2 . A thiol dependence late in the suppressor T-cell cycle would make them more similar to NK cells [212]

than most T-cells [179]. At first sight this hypothesis would seem impossible in the light of data indicating high spontaneous proliferation and IgG synthesis in RA from PBMNC, which would be occurring at physiological concentrations of IL-2, suggesting that proliferation must be occurring via a properly formed high affinity receptor (not by P75 working in isolation) and that -SH oxidation does not inhibit the ability of P75 and P55 to interact by disulphide cross-linking to form the high affinity receptor: however, one could envisage that RA cells have become activated for some unknown reason and are expressing high affinity IL-2 receptors, and one could speculate that these are disulphide linked, and under oxidising conditions the receptor is kept locked in this form preventing its the down-regulation which is vital for the normal regulation of the immune response [213]. Since the high affinity receptor has a k_d of 10 pM (unlike the LA receptor with a k_d of 10 nM or the IA receptor with a k_d of 1 nM) and takes advantage of the fast binding capacity of the β chain and slow releasing capacity of the α chain, it is able to be stimulated by very small amounts of IL-2, so one would not need disturbances in IL-2 production for uncontrolled immune responses to occur, and locked in this form one could imagine an uncontrolled immune response causing B-cell differentiation and proliferation to IgG synthesis and uncontrolled T-cell proliferation. One could envisage that sulphhydryl drugs re-reduce the high affinity receptor, thus allowing the down-regulation of the immune response.

We suggest that experiments need to be carried out to directly answer the question as to whether the high affinity receptor is a

disulphide-linked heterodimer and whether PHMPSA treatment prevents cross-linking. It has been shown that ^{the} β chain can form disulphide-linked homodimers able to bind two molecules of IL-2, suggesting both chains must have thiol groups. Other authors have shown that HuT [215] and human [216] Tac antigens contain disulphide intrachain bonds. α -chains have not been shown to form disulphide-linked homodimers suggesting that they do not contain SH groups [214]; however this could be due to steric hindrances preventing homodimer formation, but the α chains may be able to associate with the β chains. It is very likely that α and β chains are physically linked in view of the fact that anti-TAC mab inhibits proliferation via high affinity receptors but only binds to the β chain, and in view of the fact that the tripartite complex of α , β , and IL-2 is internalised, supporting a view of a stable association of these proteins in the HA receptor [217]. Disulphide linked heterodimeric receptors are common findings in immunology, e.g. MHC class II antigens, Ti of the T-cell receptor, etc.... One method to examine this possibility would be to compare SDS gel electrophoresis bands after T cell activation. Under reducing conditions (2-ME) followed by alkylation to prevent S-S bonds reforming, the cross-linked dimer (if present) would appear as two single bands representing α and β chains respectively, whereas under non-reducing conditions the heterodimer would remain as a single high molecular weight band. To confirm the disulphide cross-linking one could pre-treat cells with PHMPSA before mitogen stimulated activation and compare the bands obtained under reducing and non-reducing conditions again: if cross-linking is involved in high affinity receptor

formation then, following PHMPA treatment under both reducing and non-reducing conditions, two single bands should be visible.

We also suggest that Mabs need to be developed specifically to P75 to establish whether PHMPA treatment affects P75 expression or induction (since resting cells have 600 - 700 intermediate affinity sites, but activated cells have 2000 - 4000 high affinity sites if uniformly distributed on resting cells, suggesting a 3 - 6 fold increase in intermediate affinity receptor [203], if however the P75 is not uniformly distributed on resting cells it might imply that it is selectively expressed on only a defined subset of resting cells and absent on others), or ability to bind IL-2 or transduce signals. Whilst Mabs to human P75 remain unavailable, NK cells that express the α chain in the absence of β chains (2300/cell) [218,219] may prove useful in experiments designed to look at the thiol dependence of IL-2 binding and transduction mechanisms from P75. In particular, experiments need to be designed to see if -SH containing G-proteins are involved in the activation signal.

Recently the subject of T-cell suppressor activity has become a controversial area, and a number of papers by some eminent immunologists have been written speculating even on the very existence of T_S cells: in particular they questioned the existence of Ag-specific T_S cells, about which certainly less is known. The controversy regarding Ag-non-specific T_S cells relates in particular to the fact that it may represent an in vitro artifact for reasons already described with respect to the CON-A induced suppressor assay. One of these arguments - i.e. the possibility that the inhibitory

phenomenon could be due to IL-2 consumption - has also been used to cast doubt on various other in vitro assays, including allo-Ag-specific T_S cells, spontaneous T_S cells and perhaps even SLISA, in fact any in vitro assay where T_S cells may be proliferating and expressing CD25. Further reasons for disbelieving the existence of Ag-specific T_S cells have been given by Möller [220]. He questions the assumption that Ag-specific T_S cells are a separate T-cell subpopulation for the following reasons: 1) there is no marker for distinguishing suppressor from cytotoxic cells: thus, a pure suspension of T_S cells has never been seen; 2) the suppressor I-J gene cannot be found in the place where it has been mapped; 3) Ag-specific T_S clones or hybridomas have nonsense rearrangements of genes for the T-cell receptor, or else the genes are totally deleted: in contrast T_H and T_C clones have functional genes for the T-cell receptor. Other reasons include the everchanging and fashionable attributes ascribed to suppressor factors; the immense complexity of these cells, factors and interactions postulated; and the lack of knowledge about nearly every important characteristic of T_S cells, e.g. specificity, MHC restriction, frequency of reacting cells, mechanism of suppression and target of suppression. Others, in reply to these statements, suggested that phenomena observed in various experiments should not be dismissed simply because of our present lack of understanding in this field. These workers put forward the following explanations for some of the confusing experimental results to date, and give some very good reasons for believing in the existence of T_S cells [221-225]. In brief they suggest that 1) T_S cells show unique activation requirements that distinguish them from T_H and T_C cells, i.e. antigen non specific, T_S

cells require suboptimal doses of mitogen whose presence must not be long lasting and alloantigen and antigen specific T_S cells require optimal or supraoptimal doses of antigen for optimal activation, they show unique indicator culture requirements, e.g. superoptimal responder cell numbers for optimal antigen-specific suppressor expression and suboptimal responder cell numbers for optimal non-specific suppressor expression; 2) some T_S cells do show MHC restriction, e.g. $T_{S/I}$ cells are restricted by HLA-DQ; 3) T_S cells show a bias toward particularly high connectivity; 4) T_S cells do preferentially express certain markers, e.g. CD11 on $CD8^+$ cells and CD45R on $T_{S/I}$; 5) T_S cells probably do not have a unique surface Ag, since, like interleukin production, suppressor activity is probably the result of a variety of cells with different phenotypes; 6) only for those T_S cells where MHC restriction is claimed must an α - β TCR be found: interestingly a dimeric S-S linked 90 kd protein has been found in mouse monoclonal antigen-specific suppressor T-cells [226]; 7) if you only regard a T-cell as bearing a T-cell receptor, then no T_S cell exists, however it could be that a distinct set of rearranging families - as yet unknown - exist for their T-cell receptor; 8) it may not be necessary for T_S cells to have T-cell receptors since $CD4^+$ - $CD8^+$ interactions could involve idiotypic recognition of Ag fragments on $CD4^+$ or an anti-idiotypic recognition by T_S of $T_{S/I}$ T-cell receptor, thus avoiding the need to directly interact with Ag; 9) their existence is inferred from the fact that one cannot explain the behaviour of the immune system in all situations solely on the basis of B, T_H , T_C and monocytes alone; 10) the I-J molecule is now regarded not as a suppressor marker but is thought to be adaptively acquired

and to represent an idotype of the T cell receptor, so T_S cells may not recognize antigen but an idotype on T_H T cell receptors which is antigen specific; 11) T_S cells are not one category of cells but like the T_H cells represent a heterogeneous category comprising cells showing antigen specific genetic restrictions, non antigen specific functions, differing cell surface markers and differing effector functions; 12) the main problems initially found in getting clonable T_S cells was simply because CD8 cells when cloned were T_C cells, however it is now possible to clone T_S cells which behave suppressively in a non cytotoxic fashion in an IL-2 containing medium with autologous antigen primed CD4, $T_{S/I}$ feeder cells; 13) T_C and T_S functions may represent different states of activation of CD8 cells, T_C cells being virgin cells, and T_S being memory cells as occurs in B and CD4 populations; 14) it is suggested that there are too many examples of suppressor phenomena in vivo in animal models to question the existence of the phenomenon itself; 15) a recent piece of evidence providing a strong argument against the currently fashionable criticism of the existence of T_S cells comes from Kölsch [227], who has successfully induced mouse antigen-specific T_S cells with low dose antigen, which could be successfully cloned and expressed α - β -TCR, and showed I-J or I-E restriction, expressed these antigens and did function suppressively in vitro and in vivo.

The challenge for the future is to provide satisfactory proof of their existence and plausible explanations for the mechanisms of nonantigen specific, and certainly of more biological significance, antigen specific suppressor cells.

Although since the 1970's research on autoimmune disease has been dominated by the idea that autoimmunity happens in an otherwise normal human being because of defective regulatory T cell circuits, the result of defective number and/or functions of T_S cells, one could question whether this is the correct view for the following reason: one would expect that a generalised non specific reduction in number and functions of T_S cells would be followed by simultaneous responses to many different autoantigens, and this does not happen. Autoimmune diseases are usually based on immune responses to single autoantigens or are tissue specific, and when two autoimmune diseases are present in one individual there is usually a time lag in the onset between the two. The autoantibodies that result are restricted in specificity and idotype, suggesting that there is not a generalised breakdown of the tolerance mechanism, but a highly specific abnormality in the immune system; possibly in only certain suppressor systems or perhaps even at the level of B cells making the autoantibodies or even excess activity of $T_{S/I}$ cells. As such, a recently proposed alternative view is that autoimmunity could occur in a genetically predisposed individual containing pre-forbidden pathological clones which eventually under certain environmental conditions form forbidden clones with receptors reactive to certain auto antigens [228].

Chattopadhyay [230] found in a patient with X-linked hypogammaglobulinemia and arthritis there was excess spontaneous suppressor activity in the SF lymphocytes and blood and no T_H activity. He suggested that RA patients who are hypogammaglobulinemic may have raised $CD8^+$ cell numbers or activity, whereas those showing

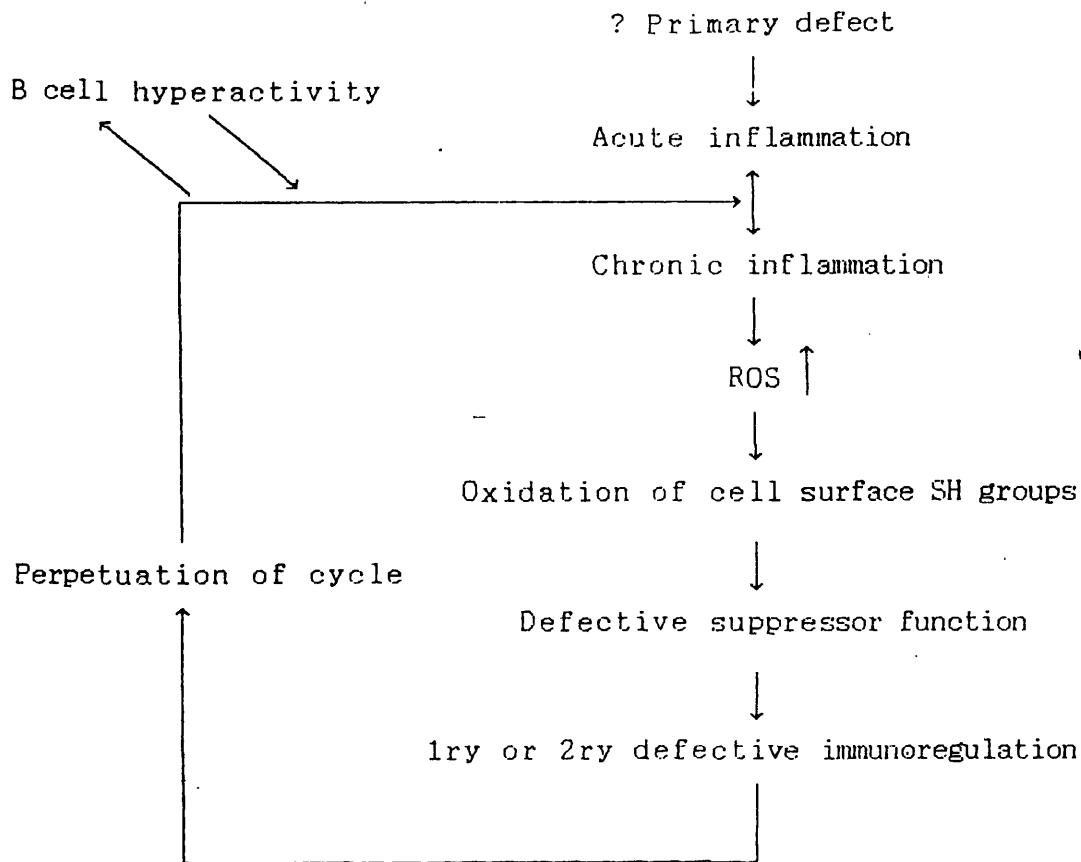
hypergammaglobulinemia may have low CD8⁺ cell numbers and activity. Since arthritis was present even in these patients with excess suppressor activity, it may be hypothesised that having suppressor activity does not necessarily protect an individual from joint problems. Neither can it be said that absence of suppressor cells is a "sine qua non" for production of arthritis, since patients can be found who are hypogammaglobulinaemic and arthritic, and therefore with suppressor cells [229]. Therefore, lack of suppressor activity may not be a primary defect, but could determine the chronicity of joint inflammation and damage. Longitudinal studies are needed to ascertain if defective suppressor activity leads to worsening joint problems.

CHAPTER 5.2 - CONCLUSION

The aim of this project was to assess what aspects of immunoregulation may be mediated by cell surface sulphhydryl groups. As such, this project has shown that serum thiols are reduced in RA-NSAID and that CON-A induced and short-lived suppressor activity are defective in PBMNC from patients with RA-NSAID and other diseases associated with excessive inflammation and dysfunction of the immune system, e.g. SLE. However, the CON-A induced suppressor activity is not defective in rheumatic diseases associated with inflammation and only mild or no immune dysfunction, like AS and PSA.

2-ME treatment in vitro improved defective CON-A induced suppressor activity in RA NSAID and RA patients on sulphydrate drugs showed improved suppressor activity in both assays.

PHMPA and H_2O_2 inhibited CON-A induced suppressor cell activity in a dose dependent manner, suggesting that oxidation of cell surface SH groups in vivo may be one factor to account for the defective activity in RA-NSAID, thus the hypothesis diagrammatically depicted on the following page was proposed. The cycle could be self-perpetuating in view of the fact that certain T_S cells may themselves regulate a ROS production [231].



Whether present as a primary defect leading to the development of the autoimmune status or secondary to disease activity, it can clearly be seen that impaired suppressor cell activity due to cell surface SH oxidation could play an important permissive role by allowing the expression and/or perpetuation of damaging immune reactions directed ultimately against the synovial joints, such as cytotoxic reactions against synovial cells, excessive T_H and monocyte and neutrophil activity, loss of tolerance and B cell hyperactivity.

To explain the inhibitory effect of PHMPSA on CON-A induced

suppressor "activity" but not "induction" and yet its inhibitory effects on CON-A induced proliferation, it is hypothesized that proliferation is probably not necessary for suppressor cell "induction" or "activity" in a PWM stimulated Ig producing indicator system, and that via an unknown mechanism independent of IL-2 and IL-2 receptors, only suppressor "activity" is -SH dependent. This might happen via PHMPA inhibition of induction, expression, binding or signal transduction from transient T_S receptors that appear later in the cell cycle. It also suggests that different aspects of cell activation by this mitogenic lectin have distinct SH group involvement.

Results from "educated" cells and mitogen-stimulated PBMNC suggest that early events in the T-cell cycle may not be -SH dependent. These events include competence signals for activation and growth (i.e. IL-1, IL-2 and γ -IFN production, also CD25 and HLA-DR expression), suppressor cell induction and initial signal transduction mechanisms from P75 for growth stimuli. However, events later in the T cell cycle, like progression signals for proliferation, may be thiol dependent, and they may include signal transduction via P75 for proliferation.

To explain hyperproliferation of T- and B-cells in RA in vivo despite oxidative conditions, it is proposed that the high affinity IL-2 receptor may be disulphide linked and "locked" in this form, therefore unable to undergo down regulation. Activated monocytes and contaminating activated neutrophils in mononuclear cell preparations,

spontaneously producing inhibitory ROS and prostaglandin could partly account for defective mitogen-stimulated suppressor cell production, reduced IL-2 production and defective proliferation and Ig production in RA. Alternatively, excess sensitivity of RA cells to inhibitory effects of these agents combined with intrinsic defects in T-cells could account for these phenomena.

Other immunoregulatory functions that appear to be dependent on cell surface -SH groups included proliferative responses to PHA, CON-A, PWM and anti-CD3 mab; they also included PWM stimulated Ig secretion from B cells, FMLP stimulated neutrophil $O_2^{\bullet -}$ production and IL-2 binding to its high affinity receptor, but the latter only to a slight extent.

Other immunoregulatory aspects that appear not to be dependent on cell surface -SH groups include the expression of various constitutively expressed surface antigens (CD2, CD3, CD8, CD4, CD45R, CDW29, CD57, CD11, HLA-DR and CD37); they also include the induction and expression of the activation antigens HLA-DR and CD25, and finally the production of IL-2. It was suggested that the production of IL-1 and γ -IFN was not -SH dependent since CD25 and HLA-DR antigen induction respectively were unaffected by H_2O_2 or PHMPA treatment.

The parallel effects of H_2O_2 and PHMPA and their selectiveness in inhibiting only certain cell functions suggest that only specific aspects of immunoregulation appear to be mediated by cell surface -SH groups.

CHAPTER 5.3 - FUTURE AIMS

To refute or confirm the hypothesis proposed in this thesis, one would need to show a correlation between reduced suppressor cell activity, low membrane -SH groups and raised background ROS production in individual RA patients. One would need to correlate normalisation of membrane -SH groups with improved suppressor activity and reduced background ROS production in longitudinal studies of RA patients on sulphhydrate treatment.

Experiments need to be carried out to assess whether 2-ME treatment in vitro improves defective SLSA in RA-NSAID.

Investigations are required to assess what specific membrane proteins, glycoproteins or lipoproteins interact with PHMPSA and cause subsequent inhibition of cell function. It is also necessary to decipher what role they play in cell function. It is necessary to assess whether -SH blockade or oxidation affects the functions of immunoregulatory important surface antigens, like CD4, CD8, CD45R, etc, and the up-regulation of expression of CD4 and CD8 antigens which can occur under certain circumstances.

Experiments need to be carried out to assess whether using a variety of in vitro suppressor assays in the same RA individual and using cells from different compartments, like blood, SF and SM; defects occur simultaneously in various systems and in various

compartments. Better still, one needs to detect and purify the autoantigens responsible for the development of the autoimmune RA disease, and then use them to develop in vitro suppressor assays in order to monitor the regulation of immune responses to such antigens. Such assays would be more relevant to RA and would be useful for testing novel pharmacological anti-rheumatic agents.

One should use $CD3^+CD8^+$ or $CD3^+CD4^+CD45R^+$ and $CD3^+CD4^+CDW29^+$ cells obtained by "negative panning" to assess which of the different functions mediated by the 3 cell subsets (suppression, help and suppression/induction respectively) is more sensitive to -SH blockade or oxidation.

Longitudinal studies on a larger population of RA patients, followed from early synovitis onwards, are needed to assess whether defective SLISA and CON-A induced suppressor activity correlate with disease activity and/or disease duration. If suppressor defects were observed even in patients with no or mild activity, then one might hypothesize that such defects are primary (and important in the pathogenesis of RA), not secondary.

Advances in the knowledge of the suppressor cell subsets and techniques for purifying, identifying and studying them, may permit a clearer understanding of which cellular populations the suppressor defects lie in, and may allow more direct experimental approaches aimed at testing novel sulphhydrate drugs with less toxic side effects,

better efficacy, faster activity, better bioavailability of their -SH groups, and finally which are better protected from metabolic inactivation paths.

So far, conventional RA treatment is non specific and generally systemic rather than specific for the inflamed joint, and may be followed by serious complications and general immune suppression. To date, the only direct approaches to synovial joint involvement are intra-articular injections of steroids or synovectomy, but both are often followed by relapses. Since chronic inflammation is maintained as a consequence of defects in the immunoregulatory system and since suppressor cells exert their functions either by cell-cell contact or by production of soluble regulatory substances [2], it may be possible to improve local immunoregulation by administration of Ag non specific SIRS or agents such as SISST or SISSB intra-articularly. The in vivo administration of in vitro generated SIRS to young NZB/NZW mice has proved succesful in preventing the onset of SLE in these animals [233, 234]. Alternatively, Ag specific and non specific T factors, or even T_S cells themselves [232] may be employed. Such treatments may have longer lasting beneficial effects. If the defect were at the level of T_S cell activation, then perhaps suppressor activating factors [235] or $T_{S/I}$ cell administration might be beneficial. Simultaneous intra-articular injections of hyaluronic acid and albumin, together with the agents mentioned above, may be necessary to normalise the SF compartment, and consequently improve the lubricating properties of SF

and its influence on the functions of SM cells and lymphatic cells bathed in it [236].

There is a need for a wider range of therapeutically effective ROS scavengers to prevent the oxidation of functionally important cell surface SH groups.

Once suppressor and helper factors have been characterised more fully, one could assess whether functionally important -SH groups are present on these factors, and whether their oxidation results in altered activity.

One could encourage an alteration in RA "eating habits", and suggest diets rich in -SH groups to correct the reduced thiol levels, diets rich in primrose oil and fish oils to alter membrane fatty acids and therefore alter phospholipase A₂ hydrolytic products - which might reduce the production of disease promoting PGs and leukotrienes - and finally diets rich in vitamin E to prevent lipid oxidation.

One could administer anti-idiotypic Ab's to cause Ag specific clonal depletion to eliminate pathogenic lymphocytes or modulate a specific immune response. However, first the autoantigen must be identified, and since a vast number of anti-idiotypic Ab's might need to be given due to the strong polymorphism of the histocompatibility Ag's, it may be better to use Ab's directed to transiently expressed activation antigens. A possibility is the administration of anti-CD25 Mab, or IL-2 bound to a toxin together with low doses of cyclosporin-A

(which selectively kills T_H in preference to T_S cells), in order to create selective immune suppression, such as inhibition of pathogenic clones of T_H or B-cells whilst sparing T_S cells, as has already been done successfully in preventing rejection of allografts in animal models [237].

Administration of immunoregulatory lymphokines, like γ -IFN or IL-2, may prove beneficial in patients with RA to improve NK and suppressor activity.

Separation of RA lymphoid subsets is necessary in order to carry out in vitro tests to correlate phenotypic and functional characteristics, also to assess what aspects of abnormal cell behaviour are the most important and valuable to correct. This will enable the design of novel drugs, capable of switching the disease off at an early stage before appreciable damage to the joint tissues has occurred.

Although it seems reasonable to hypothesize that defective suppressor activity is a primary abnormality in RA, one must check that the defect is not secondary to defective $T_{S/I}$ activity.

One needs to assess whether defective suppressor activity is due to defective production and/or activation of SISS-T and SISS-B from T_S cells.

In view of the inhibitory effects that sulphydrate reagents (DTT,

GSH and L-cysteine) have on disulphide containing receptors - e.g. C3bi, [199,200] -, it would be interesting to look at the effects of such reagents and 2-ME on other disulphide linked receptors (Ti of T-cell receptors, CD28, CD8 and perhaps HA IL-2R). This might allow the assessment of whether the disulphide bond is necessary either to hold the two monomers in juxtaposition for production of an active binding site, or for successful signal transduction. Alternatively, as has been suggested in a recent article, agonist binding to its receptor is thought either to promote disulphide exchange reactions by breaking disulphide bonds to form SH groups and subsequently attaching itself to the active site by forming disulphide bonds via its SH groups, or it may cause cleavage of intramolecular disulphide bridges and transform the receptor into a structurally altered state capable of activating the Gs transducing protein [238].

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CHAPTER 1 - INTRODUCTION AND AIMS

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CHAPTER 5 - DISCUSSION AND CONCLUSION

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SULPHYDRYL-DEPENDENT SUPPRESSOR CELL FUNCTION IN RHEUMATOID ARTHRITIS (RA)

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Suppressor cell activity is virtually absent in rheumatoid synovial membrane (1) and may be impaired in the circulation (2). We have investigated whether these findings could be due to oxidation of membrane sulphydryl (SH) groups.

Peripheral blood mononuclear cells (MNC) were obtained from 22 healthy controls, 13 RA subjects receiving NSAID only and 6 patients with ankylosing spondylitis (AS). Suppressor activity was induced by incubation with Con A for 48 hrs and assayed in a second culture of autologous cells generating IgG in response to pokeweed mitogen. Surface SH groups were blocked with pHMPA (50µM) for 1 hr at 37° either before or at the end of the 48 hr Con A culture.

Normal MNC generated maximum suppressor activity (median 89%) after culture with 10 µg/ml Con A. Induction of suppressor activity was unaffected by pretreatment with pHMPA. However, blockade of SH groups after induction markedly impaired suppressor function (median 24%, $p < 0.001$ cf untreated). Rheumatoid MNC generated less suppressor activity in response to Con A (median 51%, $p = 0.002$ cf controls). However, preincubation with an SH-reducing agent 2-mercaptoethanol enhanced suppressor cell activity to control levels (median 82%). Con A-induced suppression by AS cells was normal (median 84%).

These results indicate that suppressor cell activity may be impaired in RA following oxidation of surface SH groups. Thus the chronic inflammatory process may contribute to local immune dysregulation in the rheumatoid synovial membrane.

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